CHANGES IN GENOME STRUCTURE AND GENE EXPRESSION IN METHOTREXATE-RESISTANT MOUSE CELLS

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ABSTRACT

Several independently-selected methotrexate (MTX) - resistant mouse cell lines and their wild-type counterparts have been characterized. In the first part of the thesis, dihydrofolate reductase (DHFR) protein, RNA and DNA have been investigated. Measurements of enzyme activity in crude cell extracts and comparisons of polypeptides by SDS-polyacrylamide gel electrophoresis show that each MTX-resistant cell line overproduces DHFR. In vitro MTX inhibition studies and peptide mapping by partial proteolysis show that no extensive changes in DHFR structure have occurred. Associated with this enzyme overproduction is an increase in the amount of DHFR-specific RNA and an increase in the number of DHFR genes. In most MTX-resistant cell lines, the structure of the amplified DHFR genes visualized by DNA blotting is identical to that seen in the wild-type cells and in mouse liver DNA. However, in three out of four MTX-resistant EL4 cell lines, extensive DNA rearrangement has occurred near the ends of the DHFR genes. This rearrangement is different in each case but always affects only a proportion of the DHFR genes in the population.

In the second part of the thesis, amplification of other DNA sequences in these cell lines has been investigated. Examination of restriction enzyme digests of total nuclear DNA separated by gel electrophoresis shows that some of the MTX-resistant cell lines possess many bands which wild-type cells lack. These bands are the same in different cells in a single population and in different amplified units in a cell, but differ in independently selected cell lines. Hybridization of DNA blots using probes of total cDNA from wild-type or resistant cells shows that transcribed sequences other than DHFR have been amplified; hybridization using satellite DNA shows that some of this sequence has been amplified; and hybridization using a rabbit β-globin cDNA probe shows that a cross-reacting sequence has been amplified and/or rearranged.

In the third part of the thesis, a model for the amplification process is proposed. There are two distinct phases. First, an amplifiable unit is constructed by ligation of random DNA fragments, in a manner similar to that seen in DNA-mediated gene transfer experiments. This process produces the observed DNA rearrangements and is the cause of the observed heterogeneity between independently-selected cell lines. Second, the intact unit is amplified in the population by unequal segregation followed by selection of those cells with increased numbers of copies.

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CONTENTS

CHAPTER 1

INTRODUCTION

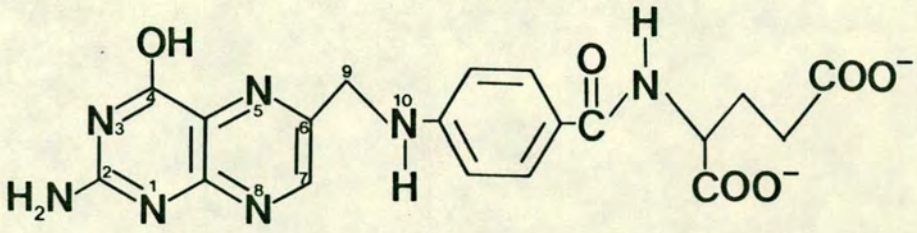
1.1. GENERAL

Methotrexate (MTX) is one of a group of folic acid antagonists. These compounds are of interest primarily because of their use in cancer chemotherapy. Cancer is the cause of about 20% of deaths in the UK (1,2), and this figure is likely to be similar in most developed countries. It is difficult to find out what proportion of cancer patients are treated by chemotherapy, or what proportion of those who are treated receive MTX; never--theless it is clear that MTX is an important agent. For example, Livingston and Carter (3,p132) write:"it is one of the most useful anticancer agents so far developed, and one of the most commonly employed". However, its usefullness is limited by the poor response of the more common lethal tumors (lung,breast and colorectal), and by the development of resistance in those tumors such as the leukemias which are initially responsive. The tumors for which MTX is never effective are probably inaccessible to the circulating drug;for a discussion see (4). However, the resistance acquired after drug treatment, leading to the relapse of a patient after an apparently complete regression of the tumor, is a different problem. This kind of acquired MTX resistance has been investigated in animals, chiefly mice and hamsters, in the hope that understanding it will lead to more effective therapy. In this thesis the characterization of a number of MTX resistant mouse cell lines is described.

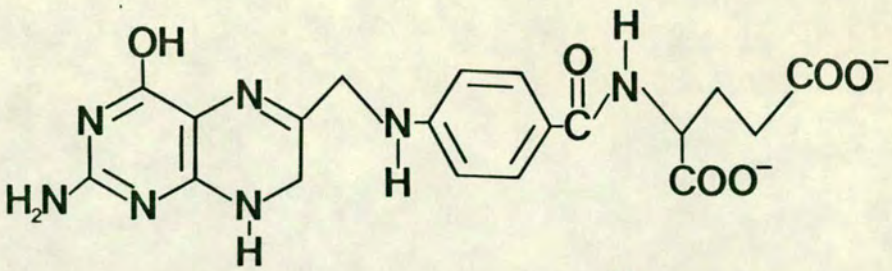
The arrangement of the introduction is as follows. First the origin and clinical use of antifolates are described; then the basis for their effects; and finally the mechanisms by which resistance to them can occur.

a. Folates:

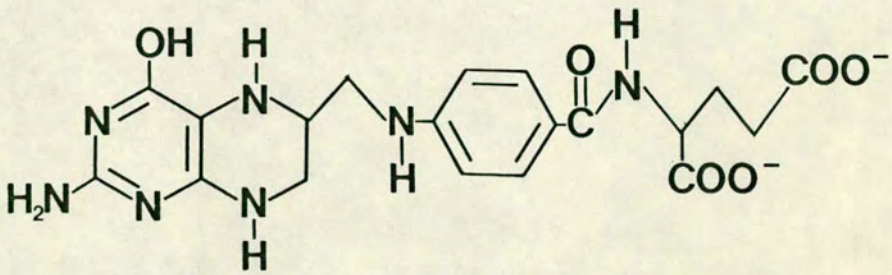
folate:
(F)



dihydro-
folate:
(DHF)

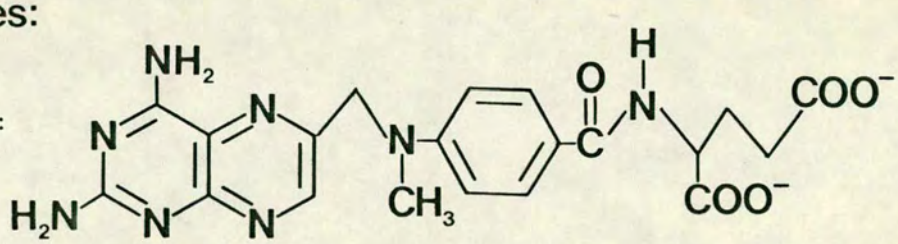


tetrahydro-
folate:
(THF)

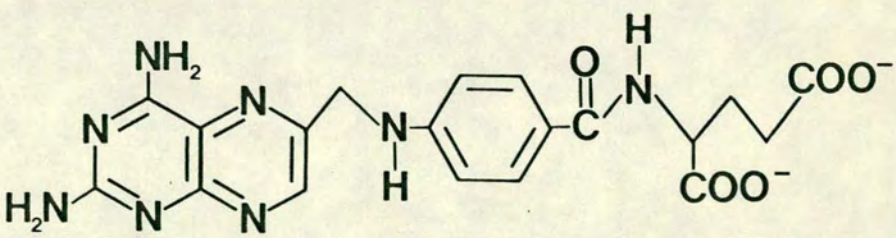


b. Antifolates:

methotrexate:
(MTX)



aminopterin:



methasquin:
(MQ)

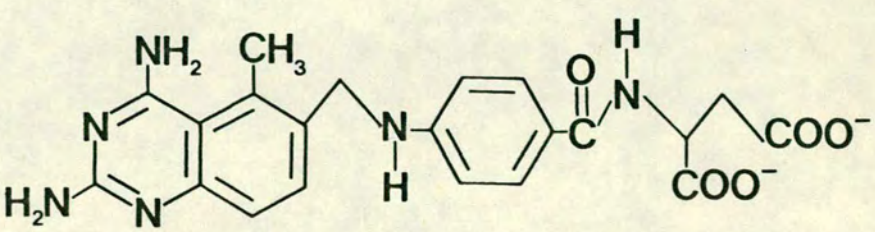


Fig. 1.1. Structures of folates and antifolates. Part of the numbering system for folate is shown.

1.2. THE CLINICAL USE OF ANTIFOLATES

1.2.1. The origin of antifolate therapy

Antifolates have been used in chemotherapy since the late 1940 s. The story of their introduction is confused by the complex nature of folates from biological sources and by the general absence of essential experimental detail from the literature.

In 1944, workers at Lederle (5) reported the isolation of a compound which stimulated the growth of Lactobacillus casei and Streptococcus lactis R, and which they therefore called 'L casei factor'. They did not report the source of the compound or the isolation procedure. 'L casei factor' was obtained by Leuchtenberger et al (6) from Lederle and tested for antitumour activity. After 6 weeks of treatment it produced "complete regression" in 43% (38 out of 89) of mice from three strains with spontaneous breast cancer. However, the following year complications appeared when this group reported that 'L casei factor' from liver was not effective, although they found that 'fermentation L casei factor' was effective (7).

Farber has described (8) how, on the basis of these results, he treated 11 children with acute leukemia with pteroyltriglutamic acid (folic acid diglutamate) synthesized by Lederle (9), which was thought to be 'fermentation L casei factor'. It led to an "acceleration phenomenon": ie. it caused the children to die more quickly. This suggested to Farber that an antagonist of folic acid might prolong the lives of his patients. Initial work with pteroylaspartic acid and methylpteroic acid was encouraging,

and when aminopterin became available in 1947 (10) Farber et al (11) found that it produced temporary remissions in 10 out of 16 children with acute leukemia: two of these were still alive after more than a year (8). These results were spectacular and unprecedented, and must be compared with the frequency of spontaneous remission which is low (about 10%) and has an average duration of about 10 weeks (8). Other clinicians found beneficial effects of aminopterin in the treatment of acute lukemia in children (12), although in adults with chronic or acute lukemia the first results were either negligible (12,13) or slightly beneficial.(14)

The relapses in patients treated with diglutaryl folic acid are, in retrospect, understandable; but it is not clear, even in retrospect, why regressions were seen in mice treated with this compound.

Subsequently, MTX has replaced aminopterin as the clinically used antifolate. On a molar basis MTX is about an order of magnitude less effective, but its theraputic index is higher: for a given toxicity level it has greater antitumour activity in mice (15) and humans (16).

1.2.2. The effectiveness of antifolates

The results that have been obtained using MTX and aminopterin on patients with a variety of tumours have been collected by Livingston and Carter (3). When considering these figures, it must be born in mind that they include the work of different practicioners in different hospitals at different times and using different dose schedules. Thus what constitutes an "evaluable" patient varies: the number of evaluable patients was usually less

Table 1.1. has been prepared from their data; the original references can be found in (3).

Tumour	Number of patients evaluated	Number of patients responding	% response
Breast	259	87	34
Colorectal	128	19	15
Lung	101	25	25
Malignant melanoma	27	3	11
Lymphoma	45	13	29
Burkitt's lymphoma	69	19	10
ALL-AUL (systemic)	159	69	40
ALL-AUL (CNS disease)	162	133	82
AML-AMoL	140	23	16
Head and neck	232	101	44
Cervix (intra-arterial treatment)	71	33	47
Cervix (systemic treatment)	25	5	20
Brain (intra-arterial)	11	4	36
Brain (intrathecal)	36	23	64
Trophoblastic malignancies	230	196	85
Testicle	10	4	40

Table 1.1. The clinical effectiveness of MTX and aminopterin against different tumour types. The data have been derived from ref 3.

than the total number of patients treated. Also, what constitutes a "response" varies: generally a greater than 50% tumor regression, ie. halving of the tumor size, was considered a response; a long term remission is not implied.

From table 1.1. it can be seen that MTX is an effective agent for some types of tumor. ALL-AUL and trophoblastic malignancies show responses in over 80% of patients, and sustained complete responses in about half this number. For most other cancers MTX is no more effective than other chemotherapeutic agents.

The dose schedules that were used include small daily doses and larger monthly doses. Some clinicians administer a single dose high enough to produce lethal toxicity, followed a few hours later by citrovorum factor (5-formyl-THF) to rescue the more sensitive tissues in the patient. Another practice, which is now general and has largely replaced the use of single agents, is combination chemotherapy: MTX is given at the same time as other classes of antitumor agent. The advantages of combination chemotherapy in clinical practice are described in reference 16.

The effectiveness of antifolates is limited by several factors. In the short term, there is an upper limit to the dose which it is possible to administer because toxic side effects occur. The most important of these are bone marrow depression and gastrointestinal ulceration. More rarely alopecia and dermatitis have been observed; when high dose/rescue therapy is used renal failure can occur. Attempts are being made to

overcome these side effects by increasing the proportion of MTX delivered to the tumor cells and therefore decreasing the relative exposure of other tissues. Kosolski et al (17) have shown that entrapment of MTX in lipid vesicles increases its chemotherapeutic activity against solid tumors in rats and mice. Alternatively, polycations such as poly-L-lysine and DEAE dextran have been used to form complexes with MTX (18): these complexes can then be taken up by some cells and may be selectively taken up by tumour cells. Neither of these methods has yet been used clinically, and it remains to be seen whether they will increase the effectiveness of MTX.

In the long term it is, unfortunately, common for tumours to develop resistance to MTX after drug treatment. This is illustrated by the ultimate relapse and death of patients who have had a remission and are on maintenance therapy. Relapses are unpredictable and their molecular basis has not been determined, although work done in animals is relevant to this question. This is discussed in sections 1.4. and 1.5.

1.3. THE BASIS OF MTX CYTOTOXICITY

MTX was synthesized as a folate analog and thus it was expected to act by interfering with folate metabolism. This expectation has been born out by all the work that has been done, and its mechanism of action is probably better understood than that of any other chemotherapeutic agent.

1.3.1. The target enzyme: dihydrofolate reductase

As early as 1950, Nicol and Welch (19) showed that aminopterin blocked the conversion of folate to citrovorum factor (CF) in slices of rat liver. When the structure of CF was established as N-5-formyl-THF, attention focused on the enzymes in the pathway:

F → DHF → THF → N-5-formyl-THF

Futterman (20), using a partially purified chicken liver enzyme, found that both the reduction of FA and DHF were inhibited by aminopterin. The following year Osborn et al (21) purified dihydrofolate reductase (DHFR) from chicken liver and showed that activity was inhibited by both MTX and aminopterin. DHFR s have since been studied from a wide range of organisms: for a review see (22). In mammals the same enzyme catalyses both DHF → THF and, at about a 10 fold slower rate, F → DHF. All known DHFRs are inhibited almost stoichiometrically by MTX: reported K_i values are about 10^{-9}M (22). A wide variety of activators are also known, including KCl, urea and p-chloromercuriphenyl sulphonic acid, but there is no evidence that these are physiologically important.

DHFR s purified to homogeneity from beef heart, pig liver, Erlich ascites cells (mouse), L1210 cells (mouse), S180 cells (mouse) and BHK cells are fairly small proteins with molecular weights in the range 20 Kd to 23 Kd (22). A size of 33.5 Kd for the calf thymus enzyme has been reported (23), but this has not been confirmed. Association of subunits has not been found and the single polypeptide chain is thought to

be the active enzyme. An N-terminal sequence for the beef liver enzyme has been determined (24,25); and preliminary (26) and revised (27) sequences for the L1210 enzyme, and the sequence of the pig liver enzyme (28) have been published. There is 89% identity between mouse and pig enzymes, indicating a fair degree of evolutionary conservation. Three dimensional structures are not known.

1.3.2. Folate metabolism

Folates have a central metabolic role as carriers of one-carbon units. Reactions involving them are essential for the de novo formation of thymidylate, purines and glycine, and also for some amino acid interconversions. The relationships of the various coenzymes, as they are described in any standard biochemistry text, are outlined in fig.1.2. It should be noted that the active coenzymes are all reduced THF derivatives; and that a supply of reduced folate is therefore essential for synthesis of proteins and nucleic acids. In consequence inhibition of DHFR blocks synthesis of these macromolecules, both through lack of precursors and coenzymes, leading to inhibition of cell growth and eventually cell death.

To this oversimplified picture must be added a number of complications. As analytical techniques have improved in recent years, it has become apparent that intracellular folates exist largely in the form of polyglutamates or "conjugates" (reviewed in (29)). These derivatives have more than one glutamate residue linked to the pteroyl ring by γ carboxyl peptide bonds.

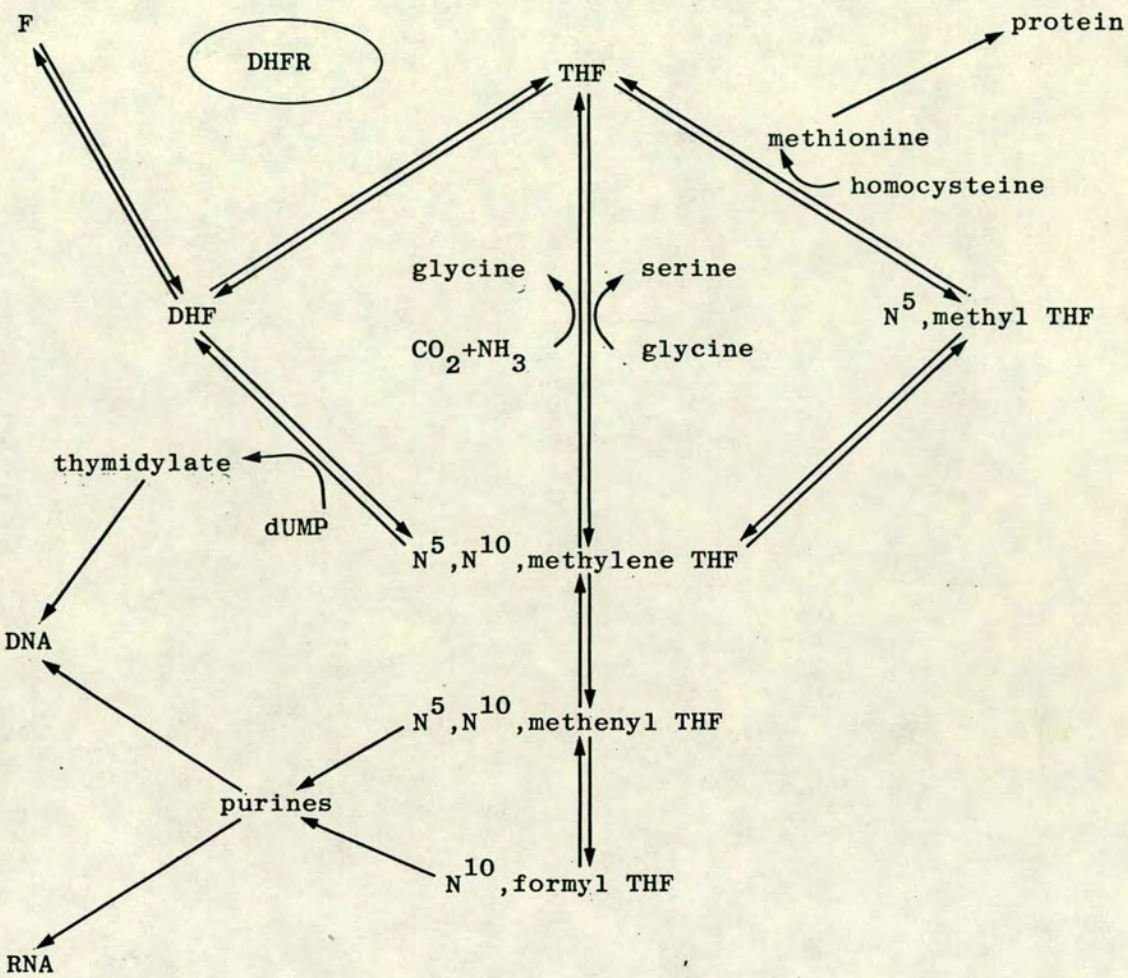


Fig. 1.2. Folate metabolism and the one-carbon pool.

Key: F = folate

DHF = dihydrofolate

THF = tetrahydrofolate

DHFR = dihydrofolate reductase

⟺ indicates an enzymic interconversion

⟶ indicates a precursor → product relationship

Note that the nomenclature of these compounds is confusing: for example, THF-diglutamate (abbreviated here THF-Glu_2) contains three glutamate residues.

The occurrence of polyglutamates in micro-organisms has been documented for over thirty years , and they have been identified in mammals more recently. Noronha and Aboobaker (30) showed that they were the predominant form of folates inside human blood cells, although the monoglutamyl forms were found in the plasma. Folates in human liver are polyglutamates with three or more glutamate residues (31). The number of glutamates has in some cases been precisely determined. For instance, Houlihan and Scott (32) showed that in rat liver folate tetraglutamates were the major species, accounting for 85-90% of the total folate.

In mammals there is probably a single enzyme, FPGS (folate polyglutamate synthetase), which catalyses the stepwise addition of glutamate residues: such an enzyme has been partially purified from rat liver (33). However, it is not well characterized and it is not known what determines the relative proportions of different polyglutamates. Results on substrate specificity vary: in rat, THF is a substrate but 5-methyl-THF is not (34); in sheep, THF, 5-methyl-THF and 5-formyl-THF are comparable substrates (35).

At one time it was thought that polyglutamates were storage forms of folate and that the active coenzyme forms were monoglutamates. The opinion was re-inforced by the finding that monoglutamates are active in <u>in vitro</u>

enzyme assays. Now that synthetic polyglutamates are available, however, it is apparent that the polyglutamate forms are the more active. This has been shown *in vitro* for human and mouse DHFR (36), human thymidylate synthetase (37), bovine methionine synthetase (38) and chicken AICAR transformylase (39). *In vivo* evidence for the importance of polyglutamates comes from analysis of a mutant CHO cell line isolated by McBurney and Whitmore (40) which is auxotrophic for glycine, adenosine and thymidine (hence GAT^-). Normal CHO cells contain mostly THF-Glu_4 derivatives, but this mutant has low intracellular folates which are mostly monoglutamates. Extracts of GAT^- cells have less that 3% parental FPGS activity (41).

Thus fig.1.2. must be modified by assuming that active folate derivatives are all polyglutamate forms with varying numbers of residues.

1.3.3. Does DHFR inhibition account for MTX cytotoxicity?

It is clear that MTX strongly inhibits DHFR *in vitro*. But does this inhibition account for its cytotoxicity? In this subsection I will consider two topics: *in vivo* modifications of MTX, which might alter its properties; and other cytotoxic effects of MTX.

As analytical techniques have improved, an increasing number of metabolic products of MTX have been identified and studied. Polyglutamate derivatives have been found widely when they have been looked for (29). For example, rats treated with MTX formed MTX-Glu_1 and MTX-Glu_2 in their liver and kidneys (42). These were rapidly turned over: MTX-Glu_1 disappeared at 8 times the rate of MTX. In contrast, patients who had received MTX as a therapeutic agent accumulated and retained MTX-Glu_1 in their livers,

where it comprised between one third and all of the MTX (43). MTX polyglutamates have also been observed in cultured cells: eg Rosenblatt et al (44) found that in human fibroblasts incubated with MTX, they accounted for about 25% of the total intracellular MTX after 8 hours.

Derivatives with more than three glutamate residues have not been reported: this may be because MTX in the cell rapidly becomes bound to DHFR and is not then available for conjugation (29). Two lines of evidence suggest that MTX-polyglutamates are formed by FPGS. Firstly, MTX is a substrate for the partially purified enzyme (33); and secondly, GAT^- cells do not synthesize MTX-polyglutamates (45).

Some of these derivatives have been shown to inhibit some DHFR s as effectively as MTX itself, and this may be true of all derivatives. MTX-Glu_1 is as, or more (depending on the pH) potent an inhibitor of L1210 DHFR <u>in vitro</u> (46); and the same has been found for partially purified human lymphocyte DHFR (43).

Thus, synthesis of polyglutamate derivatives of MTX is a major occurrence but does not significantly alter DHFR inhibition. However, <u>in vivo</u> cytotoxic effects may be altered because of resulting changes in MTX retention by the cell.

A second form of MTX modification is removal of the glutamate residue to produce 2, 4-diamino-N(10)-methylpteroic acid (DAMPA), and this compound has been detected in concentrations greater than or equal to MTX in some patients (47,48). Its origin is unclear since no mammalian carboxypeptidase G is known; such an enzyme with a high

affinity for MTX has been found in bacteria (49) and it has been suggested that cleavage is carried out by gut flora (50). It is not known whether DAMPA inhibits DHFR.

A third form of modification is oxidation of MTX to 7-OH-MTX; this occurs to varying extents in different species. This product has been detected in the urine of human patients and Rhesus monkeys (51) treated with high doses of MTX, and can form up to one third of excreted MTX. 7-OH-MTX is about 2 orders of magnitude less effective as an inhibitor of DHFR (52); hence this is a significant detoxification mechanism. In rabbits, where the 7-hydroxylation reaction is important even at low doses of MTX, the reaction is catalysed by the hepatic metalloflavoprotein aldehyde oxidase system (53). It is not known whether it is this system which is active in primates.

Thus modifications of MTX occur extensively and are important in determining its toxicity <u>in vivo</u>; their importance in cell cultures is at present unclear. In particular, 7-hydroxylation has not been investigated in cultured cells.

The second question to be discussed in this section is whether MTX has cytotoxic effects in addition to its inhibition of DHFR. <u>A priori</u>, it might be expected to bind to other folate-utilizing enzymes because of its structural similarity to folate. Claims have appeared in the literature for detection of such effects; the difficulty is to distinguish direct effects of MTX from indirect effects due to DHFR inhibition and the resulting depletion of folates; this subject is controversial.

One line of investigation has been to examine the intracellular binding sites of MTX (54). When cells loaded with MTX are resuspended in MTX-free medium, part of the MTX leaves the cell rapidly (with a half-time of about 2 minutes), and part is retained (>one hour). These parts are referred to as the exchangeable and bound pools respectively; the bound pool is assumed to be bound to DHFR. Goldman (54) has provided evidence for the importance of the exchangeable pool in inhibiting DNA synthesis. Cells which had the bound pool saturated with MTX synthesized DNA at 75%+-10% of the rate of cells not treated with MTX. An exchangeable pool of at least 0.2uM MTX was necessary to reduce DNA synthesis to 50%. Goldman therefore suggested that saturation of low affinity binding sites was necessary for MTX toxicity. Folate using enzymes are candidates for these sites.

In a subsequent paper, however, White and Goldman (51) provided evidence that DHFR itself is the source of the low affinity binding sites. They found that when the bound pool was saturated, conversion of ^{3}H DHF to THF was not significantly affected; an exchangable intracellular MTX level of about 0.15uM was necessary to reduce the THF level to 50%: this is comparable to the level required to inhibit DNA synthesis to the same extent. In contrast, folic acid reduction was more sensitive: saturation of the bound pool reduced the folic acid DHF conversion to 15%. The significance of the apparently different DHFR affinities is obscure: Multiple species have not been found in wild-type cells (except as artefacts - see (22)). Possibly there are

different intracellular compartments.

Borsa and Whitmore (56) have proposed that MTX has other site(s) of action on the basis of their study of growth inhibition in L cells. Inhibition could be reversed by supplying the cells with thymidine plus adenosine, or (competitively with MTX) by 5-formyl-THF. The site of competition in the latter case could either be at the level of entry into the cell, or somewhere beyond the DHFR block (since DHFR-MTX association itself is noncompetitive). To distinguish between these possibilities, Borsa and Whitmore measured the reversal of inhibition by 5-formyl-THF in the presence of an excess of either deoxyadenosine or thymidine. When excess deoxyadenosine was used (ie. growth dependent on endogenous thymidylate synthesis), reversal was competitive. When excess thymidine was used (growth dependent on endogenous purine synthesis), reversal was noncompetitive. They interpret the difference as indicating that entry into the cell is not the important factor, and the competitive nature of the reversal in the presence of excess deoxyadenosine as indicating a site of action for MTX along the pathway for the synthesis of thymidine.

The key candidate enzyme is thymidylate synthetase (TS), and in a second paper (57), Borsa

and Whitmore claim that this enzyme is directly and competitively inhibited *in vitro* in partially purified preparations. It is clear that TS will bind MTX, at least in the presence of dUMP (its natural substrate), since it can be isolated on a MTX affinity column (58). However, other workers have found that inhibition is not competitive with respect to MTX (58); also the *in vitro* K_i is too high by a factor of at least 100 to account for the inhibition observed by Borsa and Whitmore in their cultured cells. However, if the intracellular levels of MTX in resistant cells are higher, MTX could start to cause significant inhibition in TS. This will be discussed later.

A more indirect role for TS has been proposed by Moran et al (59) on the basis of their growth inhib--ition studies. They found that inhibition by MTX of cultured mouse or leukaemic cell growth was reduced by 5-fluoro 2'deoxyuridine (FdUrd), hypoxanthine (H) or thymidine (dThd). They suggested that there was a common underlying mechanism: direct (FdUrd) or indirect (H and dThd) inhibition of TS. Reduced levels of thymidylate synthesis would reduce depletion of THF derivatives and therefore require less DHFR activity. While such a mechanism might facilitate cell survival and subsequent rescue from MTX inhibition, it is paradoxical that it should facilitate cell growth. Thus, although it is possible that MTX has modes of action *in vivo* other

than DHFR inhibition, none has been convincingly demonstrated.

1.4. MTX RESISTANCE

MTX resistance has been widely studied in cultured mouse and hamster cells, and to a lesser extent in human cells. I do not propose to review all the papers published about or using MTX-resistant cells, but have selected some of the more pertinent work. A summary of the work of Schimke's group has appeared (60) but there is no recent general review of the field.

1.4.1. Mechanisms of resistance

Resistance to MTX has been found to arise by three main types of mechanism: 1. reduction of MTX transport into the cell, so that in spite of a high extracellular concentration, the intracellular concentration never becomes high enough to inhibit DHFR significantly. 2. Alteration of the binding properties of DHFR so that its high affinity for MTX is lost. 3. Increase in DHFR activity so that although at a given MTX level most of the DHFR is inhibited, the residual activity can supply the needs of the cell. Combinations of these mechanisms frequently occur. In addition, inactivation of MTX is a possible fourth mechanism, but has not been reported. The first three mechanisms will be considered in the next three subsections.

1.4.2. Resistance due to altered transport

The first identification of a transport mutant was described by Fischer (61) in a clone of mouse L5178Y cells selected *in vitro*. This clone, designated m^t, was obtained by a two-step selection procedure, the first selection being in $2x10^{-8}$M MTX and the second in a concentration not reported. L5178Y m^t cells required $5.6x10^{-7}$M MTX to inhibit the rate of cell growth by 50%; this represented about 70-fold resistance. An alteration to the MTX transport in resistant cells was demonstrated by resuspending sensitive and resistant cells grown in nonselective medium in ^{3}H-MTX. After equilibration, the intracellular MTX concentration in the sensitive cells was two to three times that in the medium, while in the resistant cells it was 0.1 to 0.2 times: this represents a 10 to 30 fold decrease in MTX transport in resistant cells, but it is not sufficient to account for all the resistance of the cell. Other causes of resistance in L5178Y m^t were not examined, nor was the basis for resistance before the second stage of selection.

Kessel et al (62) found that sublines of mouse L1210 and P288 cells resistant to MTX (selection procedures not described) had, in addition to an eight to ten fold increase in DHFR activity, four and three fold decreases in drug uptake. Another L1210 subline (63) selected *in vivo* for multiple resistance to MTX, 5-fluorouracil and 6-mercaptopurin and called L1210 (XVI_4), had a four fold decrease in the rate of MTX transport. The V_{max} value for transport

was unchanged, but the K_m was increased, suggesting an alteration in the binding of MTX to a carrier protein. Further selection of L1210(XVI_4) produced L1210(XVI_{4a}) which had a 10 fold increase in DHFR level but, unexpectedly, normal transport of MTX.

Chinese hamster cell lines with altered transport properties have been obtained by Flintoff et al (64) after EMS mutagenesis and single-step selection in $1x10^{-7}$M MTX. Such clones were 38 to 58 fold resistant to MTX, had about a ten fold lower transport rate, but had normal amounts of the normal DHFR.

Five MTX-resistant sublines of the human lymphoblastoid line W1-L2 have been selected by Niethammer and Jackson (65) in successive stages. In the early stages of selection, resistance was due to increased enzyme activity and alterations in enzyme structure, but in later stages transport mutants occured in four of the five sublines. The one of these which was studied in detail had an un--altered K_m but at least a seven fold decreased V_{max}; thus it is of a different kind from that found in L1210 cells (63).

Folates and MTX enter the cell by active transport when their concentration is low. There are probably seperate transport systems for folates and for THF derivatives; MTX enters by the latter (66). When MTX-binding fragments from L1210 plasma membranes are isolated, three major polypeptides are found (67).

These have molecular weights of 67Kd, 63Kd and 56Kd. MTX interacts directly with the 56Kd polypeptide which in turn interacts with the other two polypeptides. The molecular basis for altered entry could be a decrease in the total amount of carrier, a decrease in its affinity for MTX, or a decrease in its rate of transport of MTX. The second of these would be effective only at low MTX concentrations; the third only at high concentrations. One of the mutations in mouse cells (63) is of the second type, and it is interesting to note that it is lost on further selection to higher levels of resistance where it would confer little advantage; the human mutations (65) are of the third type and occur late in a series of stages of selection where the system is presumably operating close to its V_{max} and a decrease in V_{max} is consequently an advantage. Changes in the polypeptides of the transport system have not been demonstrated directly.

1.4.3. Resistance due to altered enzyme

Reports of the finding of multiple or new DHFR species in cells treated with MTX must be interpreted with caution because it was not appreciated until the mid 1970 s that some of the methods of analysis, Sephadex column chromatography and activity gel electrophoresis, separate DHFR with different cofactors bound, and thus give rise to artefactual multiple forms of the enzyme. Nonetheless, enzymes

with genuinely altered structure have been detected in hamster and human cells.

Biedler and co-workers have characterized such an enzyme in Chinese hamster cells. It had been noticed that a series of nine sublines, independently selected *in vitro* for resistance to MTX, seemed to fall into two groups (68). Both groups had increased DHFR activity; but for a given level of resistance, group A (my terminology) had highly increased DHFR activity and were also resistant to a comparable level of MQ, while group B had slightly increased DHFR activity and were only resistant to a lower level of MQ.

The DHFRs from the two parental cell lines, two members of group A and two members of group B were characterized with respect to amount and relative inhibition by MTX and MQ (69). Parental cell and group A enzymes were inhibited stoichiometrically by both MTX and MQ, while group B enzymes were stoichiometrically inhibited by MQ but only weakly inhibited by MTX; weak inhibition was due to weak binding. More recently (70), a comparison was made between the polypeptides from the parental cells, a line selected for resistance to MQ (71) with a group A enzyme, and a line with a group B enzyme. When cytosol proteins were run on SDS-polyacrylamide gels a single extra band (ie. DHFR) could be seen in each resistant cell line, but the migration of this band was slightly slower in the group B polypeptides, corresponding to a molecular weight of about 21Kd, rather than 20Kd in the group A polypeptides. The same phenomenon was seen when polypeptides from the *in vitro* translation of mRNA were

analysed, suggesting that the mobility difference was due to a difference in primary structure and not post-translational modification.

Another series of modified DHFRs, also from Chinese hamster cells, have been characterised by Flintoff and co-workers (64). They purified DHFR from wild-type cells, two independently isolated mutants carrying altered DHFR and a third line which carried the altered enzyme but which had been further selected for increased amount of the altered enzyme. All enzymes had similar K_m s for DHF and FA. Slight differences could be seen in pH activation profiles and thermal inactivation kinetics, but the striking difference was the six to eight fold decrease in affinity for MTX. These workers state that the wild-type and mutant enzymes have the same mobility on SDS gels, corresponding to a molecular weight of 24Kd. However, a close examination of their fig 1 reveals that their mutant (MUT_1) enzyme runs slightly faster: the reverse of the situation found by Biedler et al. The difference between the molecular weights reported by Biedler and co-workers and those reported by Flintoff and co-workers for the wild-type enzymes probably reflects a difference in the standards used and the electrophoretic conditions rather than a real difference.

An altered enzyme has also been described in human cells by Jackson and Niethammer (72). W1-L2 cells showing 170 fold resistance , and designated WR8.1, had 222 fold increased DHFR activity and were grown in 1.6×10^{-6}M MTX. DHFR from these cells had a

K_i for MTX increased about 50 times and a K_m for DHF increased about 18 times, giving a K_i/K_m ratio of about 3. When these cells were further selected for resistance in 3.3×10^{-6}M MTX, a 520 fold resistant line, designated WR9.1 was obtained; it had 234 fold increased DHFR activity. Amazingly, the K_i and K_m of this enzyme had reverted to wild-type. Membrane transport was not altered in either of these resistant cell lines. Jackson and Niethammer explain what at first sight seems a paradoxical result as follows. At the high MTX levels that the cells are growing in, DHFR inhibition is not the only factor that determines toxicity; TS inhibition must also be taken into account. Decreasing the affinity of DHFR for MTX allows more enzyme activity and hence more THF synthesis. However, if TS cannot use the THF because it is inhibited by MTX, the cell still cannot grow. Increasing the affinity of DHFR for MTX (returning it to normal) can decrease the intra-cellular MTX concentration and allow TS to function. The genetic basis of a change such as that observed in the selection of WR9.1 from WR8.1 must, in view of current ideas about the mechanism of protein overproduction (see section 1.4.4), be considered puzzling. I will return to this subject at the end of section 1.5.2.

1.4.4. Resistance due to increased DHFR activity

Increased enzyme activity has already been mentioned in the subsections on other forms of resistance. As a sole mechanism of resistance it is well documented, and it invariably occurs, alone or in combination with other mechanisms, in cell lines resistant to high levels of MTX ($\geq 10^{-6}$M). Hakala et al (73) described two derivatives of mouse S-180 cells obtained after growth in gradually increasing MTX concentrations. One subline was 67 fold resistant and had 65 fold increased DHFR activity; the other was 174 fold resistant and had 155 fold increased activity. Fischer (74) selected L5178Y cells for resistance by a two-step procedure. The clone of the first-step selection was 2.3 fold resistant and had 2.0 fold DHFR activity; the clone of the second-step selection was 16 fold resistant and had 17 fold activity. L5178Y cells selected for resistance to 1.1×10^{-3}M MTX by Courtenay and Robins (75) had about 100 fold increased DHFR; a reduction in transport was acting here as well.

An increase in enzyme activity could, in principle, be due either to production of a more active enzyme, or to a higher level of an unaltered enzyme. The latter mechanism has invariably been shown to be the case when this question has been tested. Nakamura and Littlefield (76) purified DHFR from wild-type and resistant BHK cells and demonstrated that the proteins had similar properties, including inhibition of enzyme activity by antibodies made against the resistant cell DHFR; only the amount was

different. Later (77) Hanggi and Littlefield compared peptide maps and found them to be the same. Alt et al (78) working on AT-3000, a 3000 fold resistant cell line derived from S-180 by Hakala et al (73), found a 140 fold increase in DHFR activity. Immunological precipitation with anti-DHFR antibody showed that the resistant cells had about 150 times more cross-reacting material than the sensitive. The properties of the two enzymes purified to homogeneity were the same.

These increases in steady-state amount of protein could be due to increase in the rate of synthesis or decrease in the rate of degradation. Nakamura and Littlefield (76)initially found a 20 fold increase in the rate of synthesis in their hamster cells which had a 140 fold increase in the amount of enzyme, suggesting that both mechanisms were operating. However, Haggi and Littlefield (77), using a technique less prone to artefacts, subsequently found that the rate of synthesis was about 140 times faster and that the rate of degradation was the same. Similarly, Alt et al (78) found that the proportion of soluble cell protein precipitated by anti-DHFR was 7-8% in continuously labelled cells and 6-7% in pulse labelled cells. Thus rate of synthesis is the controlling factor.

Rate of synthesis of protein will depend on the amount of mRNA and the efficiency of its translation. Chang and Littlefield (79) prepared polysomal RNA from sensitive and resistant BHK cells and translated it

in vitro in the wheat germ system. RNA from resistant cells directed the synthesis of about 70 times more DHFR per unit of total protein than did RNA from sensitive cells. Similarly, Kellems et al (80) showed that the increase in rate of DHFR synthesis in S-180-AT3000 cells could be explained entirely by an increase in the amount or efficiency of mRNA, as judged by in vitro translation in the rabbit reticulocyte system and immunoprecipitation. Thus increased enzyme activity is due to protein overproduction, and overproduction is due to an increased amount of translatable mRNA.

1.4.5. Resistance in human tumors

It can be seen from the previous part of this section that there are multiple ways in which cells can become resistant to MTX. Which are found clinically? Changes in the amount of DHFR activity have been seen by Bertino et al (81,82) in leukocytes and erythrocytes of patients treated with MTX. For example, in one patient (VM), the level of enzyme started to increase from being initially undetectable to being detectable in five days after MTX treatment was initiated, and decreased quite rapidly after treatment was stopped on day 15. This kind of response, called "induction", was seen in patients with leukemia and other malignancies. There are considerable changes occuring in the blood cells of these patients and the rise in DHFR may be due to changes of cell type, or it may be an adaptive

response of cells already present; in any case it cannot be the selection of a genetically resistant variant because both the increase and the decrease are too rapid.

Roberts, Hall and co-workers have also investigated DHFR levels in blood cells of human patients, using a more sensitive enzyme assay. In untreated patients with ALL, AGL or CGL the DHFR level was elevated by about an order of magnitude; in CGL or non-haematological malignancies it was not significantly increased. Elevation was because of circulation of immature leucocytes which would remain in the bone marrow of a normal individual. When MTX was administered to patients with ALL, a transient increase in DHFR activity was again seen. It returned to pretreatment level while the patients were in remission, and remained at this level if the tumour became resistant to MTX (83).

These same workers also studied permeability of lymphocytes from leukemic patients to MTX (84), and found a correlation between high MTX uptake and later remission following treatment with the drug. However, they did not determine whether patients who had relapsed after an initial response had developed impaired permeability.

Thus the limited data availiable do not allow any conclusion to be drawn about the mechanisms of development of resistance to MTX under clinical conditions. The only result directly relevant is the

lack of significantly increased enzyme activity in lymphocytes from leukemic patients resistant to MTX (83), and even this is of dubious significance. A slight increase was in fact found: this might become significant if more data were obtained; also it is not clear whether the variation in enzyme measurements, reflected by the large standard deviations of the quoted figures, refer to poor reproducibility of the assay or real variation between patients.

1.5. THE GENETIC BASIS OF MTX RESISTANCE

Changesin transport proteins and DHFR account for MTX resistance; but what is the basis for these changes in protein?

1.5.1. Classical genetic analysis

The techniques of classical genetics allow mutation rates and the dominant/recessive nature of a mutation to be determined. From such data on MTX-res. cells a certain amount can be deduced about the molecular basis of resistance. Flintoff and co-workers have measured spontaneous and EMS-induced mutation rates in hamster cells (85). A clone of the wild-type cells was picked and grown for approximately 60 generations under nonselective conditions. Cells were then plated in 1×10^{-7}M MTX. Three stably resistant colonies were obtained, all with alterations to enzyme structure. From this small sample of mutants Flintoff et al calculated a mutation rate of 2×10^{-9} per locus per

generation. No mutants with altered MTX transport or increased DHFR activity were seen under these conditions. In the presence of EMS, the number of mutants with altered enzyme was increased by about an order of magnitude; in addition there were about an equal number of transport mutants; but still no increased activity mutants. At first sight this lack of increased activity mutants is surprising, considering the high frequency with which they have been found in hamster cells by other groups. The explanation for this discrepancy probably lies in the increase in resistance that the cell is required to produce in a single step. When this is low (two to three fold), increased activity is the most frequent cause of resistance, whereas when it is high (10 fold), sufficiently increased activity cannot occur.

Quantative analyses of mutation rates for cells of other species have not been carried out. Nevertheless, it is possible to get an impression of the rates from the literature, and there seem to be species-specific differences. Thus it is likely that the spontaneous appearance of transport mutants in mouse cells is significantly higher than in hamster cells, (there are many reported), and the appearance of altered enzyme mutants is lower (none reported). The situation in human cells is more difficult to asess, but from the limited data available it seems that all three kinds appear at moderate frequencies. These findings are summaraised

in table 1.2.

These three kinds of mutation have been studied by somatic cell hybridization. Littlefield (86) hybridized wild-type and increased activity mutants and found that 32 out of the 35 hybrid clones examined had intermediate levels of DHFR activity. He concluded that increased activity was not due to loss of a diffusable repressor, a possibility that had been considered by analogy with bacterial control systems such as β-galactosidase in E.coli.

Flintoff et al (85) have also done somatic cell hybridization experiments. They made proline prototrophs of their MTX-sensitive cell line and ouabain resistant mutants of each of their MTX-resistant derivatives. After fusion, hybrids were selected in proline$^-$, ouabain$^+$ medium and their MTX resistance tested. The transport mutants were recessive, but the altered enzyme and increased activity mutants were codominant. These are the results expected for transport and altered enzyme mutants, and for increased activity mutants if the increase is due to constitutive overproduction.

1.5.2. Molecular genetic analysis

Mutations which lead to the production of a struct--urally altered protein are likely to be point mutations: ie. single base substitutions resulting in single amino acid changes. The drastic effects on protein structure and activity that alteration of a single amino acid can

species	altered MTX transport	altered DHFR structure	DHFR overproduction
mouse	++	.	++
hamster	.	+	++
human	+	+	+

Table 1.2. The occurrence of spontaneous mutations to MTX resistance in cultured cells of different mammalian species.

Key: ++ = many examples known

+ = a few examples known

. = none reported

have are well illustrated by the haemoglobins. However, it must be emphasised that such events have not been identified at the amino acid or DNA level in DHFR or MTX transport proteins.

Protein overproduction by means of an increased mRNA level could, in theory, be due to several causes including activation (or depression) of a promotor leading to more frequent initiation, increased stability of the RNA, or more copies of the gene. In fact, only gene amplification has been shown to accompany DHFR overproduction. This was the work of Alt et al (87). They purified DHFR mRNA from MTX-resistant S-180 cells by immunoprecipitating polysomes with anti-DHFR antibody, and then synthesized cDNA complementary to the polyA-containing RNA in the precipitate. 15 to 20% of the resulting cDNA was DHFR-specific. This was then hybridized to excess sensitive cell mRNA, and the non-hybridizing cDNA was hybridized to resistant cell mRNA. The hybridizing fraction ('DHFR cDNA') was reassociated with an excess of total nuclear DNA from sensitive or resistant cells. The rate of hybridization was about 200 times faster when resistant cell DNA was used, corresponding to a 200 fold overproduction of protein. Similarly, a MTX-resistant L1210 line with 35 fold increased enzyme activity had about 45 times the number of genes in the sensitive L1210. Subsequently, several other cell lines have been shown to have amplified DHFR genes. These will be discussed at a later stage.

At this point we will return to the discussion of the results of Jackson and Niethammer. If the DHFR overproduction in their WR8.1 is due to gene amplification, which seems likely, and overproduction in the WR9.1 is due to the same cause, then what has apparently happened has been the replacement, in a single step, of multiple copies of one type of gene by multiple copies of another. It is difficult to see how this could happen. A trivial explanation of their observations is crosscontamination or mislabelling of their cultures; another is that the cultures which, it should be noted, are not clones, are a mixture of cells overproducing normal enzyme and cells overproducing mutant enzyme at each stage; in the two lines they may only be observing a change in the predominant form in a fluctuating population.

1.5.3. Chromosomal changes associated with MTX resistance

There have been several attempts to associate specific chromosome changes with MTX resistance. This is not simple because the chromosomes of most cultured cell lines have multiple rearrangements with respect to the original animal, and also vary from cell to cell.

In 1965 Biedler et al (88) published the results of a study on L1210 sublines. Their parental L1210 had a subtelocentric marker chromosome and in 10 out of 18 resistant sublines this was altered. They concluded that there was a relationship between the configuration of the subtelocentric and DHFR activity, and suggested

MTX had a mutagenic activity. MTX is not mutagenic in the Ames test (89) although it may lead to alterations in DNA as a result of the thymidine-less state it produces.
Since this work was carried out before chromosome banding techniques were introduced, only gross changes could have been detected; the significance of the relationship they observed is unclear.

Another kind of alteration described by Biedler and Spengler is the development of a homogeneously staining region (HSR) on a chromosome (90). Of 20 MTX- or MQ- resistant hamster sublines, 13 were near-diploid and were karyotyped. Seven of these had HSRs on one of chromosomes two, four or, in one case, an unidentified chromosome. These HSR were defined by their lack of the normal banding substructure after trypsin-Giemsa staining. In addition, the HSRs C-banded, were uniformly and early replicating and were located terminally on their chromosomes. In a further study (91), cells from two of the sublines were cloned and then grown in non-selective medium. Resistance was lost gradually over a period of about a year and there was a corresponding decrease in the size of the HSR.

Another MTX-resistant hamster line has been studied by Nunberg et al (92). This line was 50,000 fold resistant and had about 150 DHFR genes. There was an HSR, again, on chromosome two; and Nunberg et al showed by <u>in situ</u> hybridization with mouse DHFR cDNA that most if not all of the amplified DHFR genes were localised in the HSR.

Judging from photographs of chromosome spreads, they estimated that the HSR comprised about 3½% of the Chinese hamster genome, which was about 1.6×10^8 bp. Thus if the DHFR genes were spread evenly along the HSR, which the distribution of silver grains seemed to suggest, the size of the repeating unit was about 1000kb.

1.6. OUTLINE OF THIS THESIS

A conclusion from section 1.4 and 1.5 is that amplification of DHFR genes can be a basis for DHFR enzyme overproduction and thus for MTX resistance. This was an unexpected finding because gene amplif--ication is not a common mechanism by which cells or organisms increase their capacity to synthesise a gene product (93). The aim of this thesis is the elucidation of the mechanism of such amplification.

The work falls into two parts. In the first part, a number of MTX-resistant mouse cell lines are examined in order to establish whether the basis for resistance in these cells is DHFR gene amplification. In the second part, other sequences which may have been involved in the amplification are investigated. In the light of these findings, some models for the amplification process are considered.

CHAPTER 2

MATERIALS AND METHODS

2.1. GENERAL

The experiments described in this thesis were carried out over a period of more than a year. During this time existing techniques were being learned and new techniques developed. In this chapter only the methods finally adopted and variations which are significant are described; preliminary experiments and insignificant variations are omitted.

2.2. ORIGIN OF THE MTX-RESISTANT CELL LINES

2.2.1. General features of the cell lines

All the cells used in this work are transformed lines originating in mouse tumours and capable of unlimited growth in culture. The MTX-resistant lines which grow in high concentrations of MTX have all been obtained by stepwise selection in increasing MTX concentrations without the use of mutagens. In general, little cloning of the cell populations has been carried out, although in some cases cells were cloned at the initial and final selection steps. The derivation of the cell lines is outlined in fig.2.1., and details are given in sections 2.2.3. to 2.2.7. below. Photographs of the living cells are shown in fig. 2.2.

2.2.2. Nomenclature

The nomenclature system usef for the PG19 cell lines (94) is extended here to include all the MTX-resistant cell lines. The cell type or subtype is followed by'MTX_R' and a suffix which is the concentration

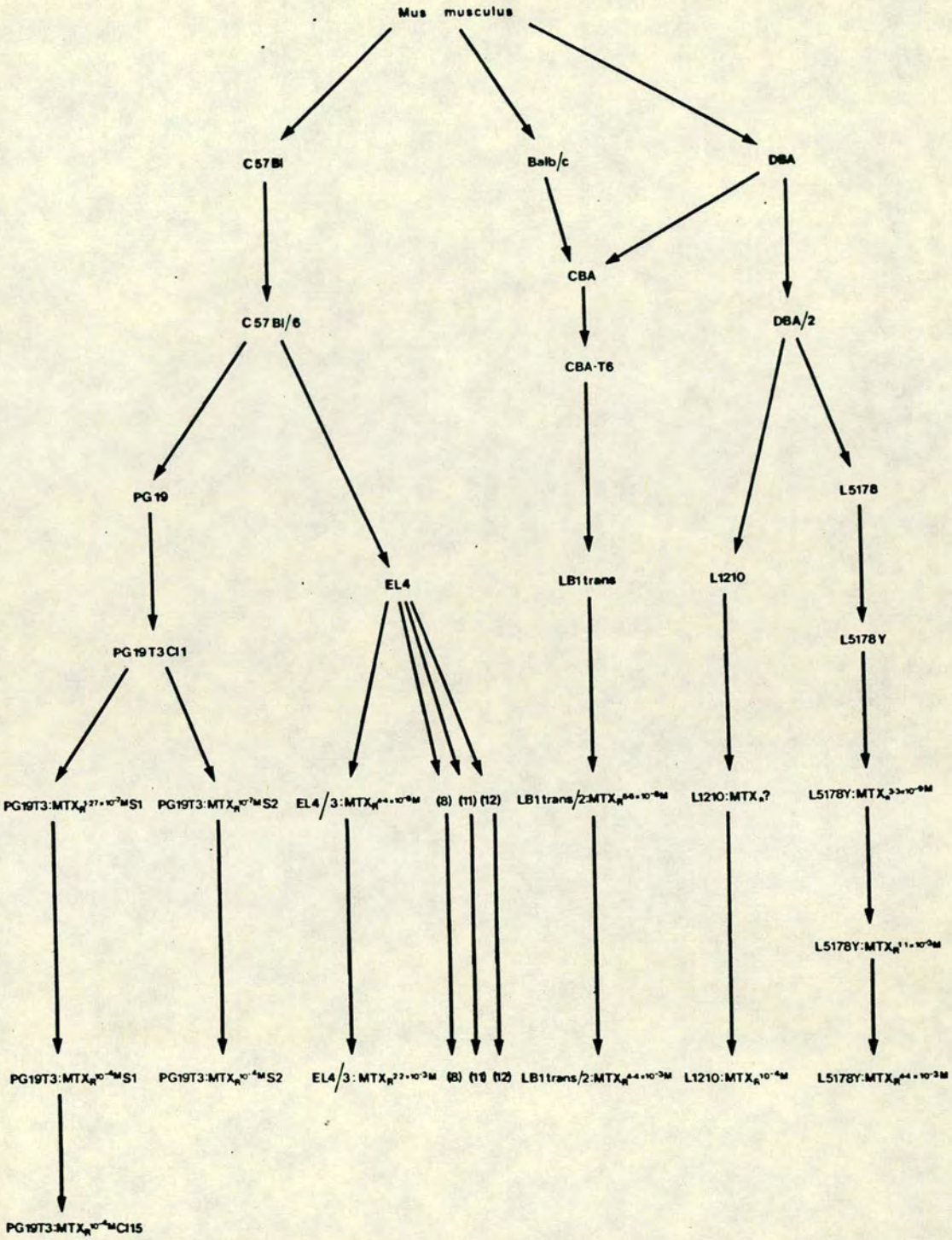
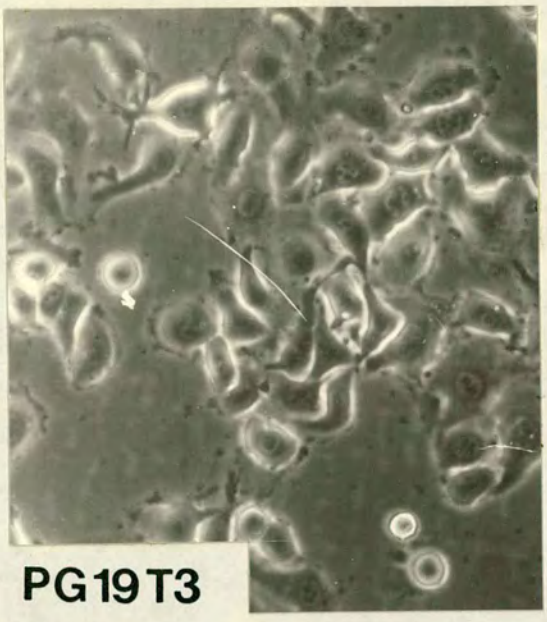
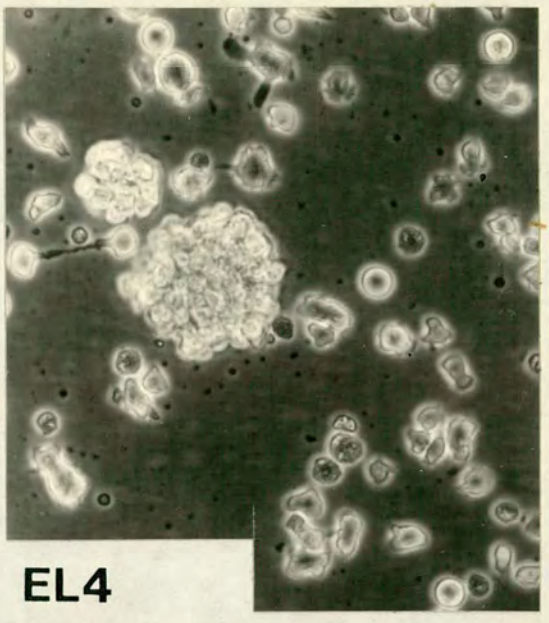
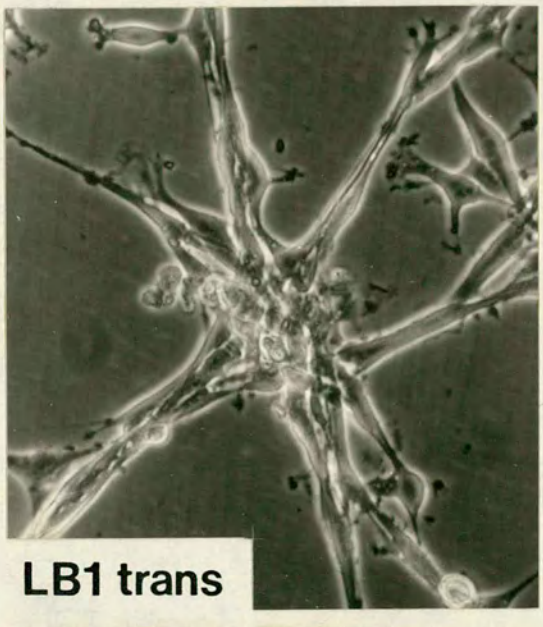


Fig. 2.1. Summary of the origins of the cell lines used in this thesis. The mouse strain and substrain, the wild-type cell line, and the initial and final levels of MTX resistance are shown.







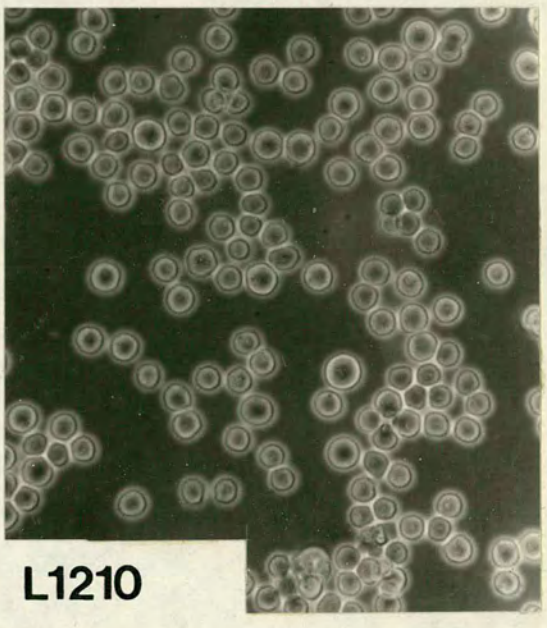


Fig. 2.2. Phase contrast light micrographs of living cells.

of MTX used for selection. Thus L5178Y:$MTX_R^{4.4x10^{-3}}$ designates a population of L5178Y cells which have been selected for growth in $4.4x10^{-3}$M MTX.

In two instances there is more than one series of MTX-resistant cell populations derived from the same starting line. The two PG19 series are designated PG19T3:MTX_R^{x}S1 and PG19T3:MTX_R^{x}S2; x is the relevant MTX concentration. The four EL4 series are designated EL4/3:MTX_R^{x}, EL4/8:MTX_R^{x}; EL4/11:MTX_R^{x}, and EL4/12:MTX_R^{x}.

In all lines the wild-type, parental or sensitive cell population is called ...../WILD.

2.2.3. PG19

PG19 cells were derived from a spontaneous melanoma in a C57B1/6J mouse (95): the melanoma cells were treated with ethyl methane sulphonate and selected for resistance to 6-thioguanine. Consequently they lack HPRT activity. Before selection for MTX resistance was initiated, the cells were passaged through a newborn C57B1 mouse; tumour cells were re-established in culture and one clone was chosen. This is designated PG19T3C11.

Two selections for MTX resistance were carried out. In both selection series S1 and S2 a clone was picked at the initial selection level; this was $1.27x10^{-7}$M MTX (S1) or $1x10^{-7}$M MTX (S2). Selection was continued by increasing the MTX concentration approximately threefold in subsequent stages. Multiple colonies were always seen: individual clones were not picked until the final stage was reached. In the S1 selection, cells growing in $3x10^{-5}$M MTX were

plated in 10^{-4}M MTX and individual clones selected. One of these clones (clone 15) was chosen for detailed study because good chromosome preparations were easily obtained from it. In the S2 selection, the population of cells growing in 10^{-3}M MTX was plated at low density in this concentration of MTX and individual clones were picked. Intermediate stages in the two selections were preserved by freezing down.

The first stages of the S1selection were carried out by V.van Heyningen and P.M.B.Walker; later stages were done by C.J.Bostock and E.M.Clark. The initial and final stages of the S2 selection were carried out by me; other stages were done by C.J.Bostock and E.M.Clark.

2.2.4. EL4

EL4 cells originated in a C57B1 mouse treated with 9, 10-dimethyl-1,2-benzanthracene in 1945 (96). and were described as a lymphogenous leukosis. Investigation of their surface antigens has shown that they are Thy-1 positive (97); therefore they are T-cell derived. EL4/WILD cells were plated in soft agar containing 4.4×10^{-8}M MTX. Resistant clones were selected and subsequently maintained separately in suspension culture. The MTX concentration was increased in two to three fold steps. The final concentration was 2.2×10^{-3}M (EL4/3) or 1.1×10^{-3}M (EL4/8, EL4/11 and EL4/12). Selection was carried out by T.Alderson.

2.2.5. LB1 trans.

LB1 trans cells were derived from a CBA-T6 mouse embryo (T.Alderson, personal communication). Initial selection was in 6.6×10^{-8}M MTX and the final selection was in 4.4×10^{-3}M MTX. Selection was carried out by T.Alderson.

2.2.6. L1210

The origin of L1210 cells has been described by Law et al (98): the skin of a female DBA subline 212 mouse was painted with methylchloroanthrene in ethyl ether, and cells from the resulting lymphoid leukemia were maintained *in vivo*. Selection for MTX resistance, carried out by Harding et al (99), was also *in vivo*. L1210 cells and MTX at a concentration of 0.007ug/g body weight were injected intraperiton--eally into BDF_1 mice. In the absence of MTX, the survival time of the mice was about eight days. MTX increased survival to 26 days because of inhibition of growth of the tumor. Some of the resulting tumor cells were injected, with MTX, into another mouse and a second-generation tumor obtained. The procedure was repeated for a total of 20 tumor generations. At the final stage the survival of the mice had decreased to eight days, the time observed when no MTX was used, and therefore the L1210 cells were considered resistant to MTX.

When these resistant cells were received in this

laboratory, they were grown in vitro in suspension culture. They were found to survive in 10^{-4}M MTX, and were routinely maintained in this concentration. Their ability to grow in higher concentrations of MTX is unknown.

2.2.7. L5178Y

L5178 is another leukemia which arose in DBA/2 mice, and the line L5178Y was derived from it (100). Development of MTX-resistant L5178Y was carried out initially by Courtenay and Robins (75) and in the final stages by T.Alderson. Selection was initiated in 3.3×10^{-9}M MTX; when rapidly growing cells were observed in the culture, the MTX concentration was doubled and this protocol continued until, after 83 days, cells survived in 1.1×10^{-3}M MTX. After maintainence at this concentration by Courtenay and Robins, selection was continued by T.Alderson to a concentration of 4.4×10^{-3}M MTX.

2.3. CELL CULTURE

2.3.1. Maintenance of cells

All cells were grown in RPMI 1640 medium (Flow Laboratories) supplemented with 10% heat inactivated foetal calf serum (Gibco Bio-cult), antibiotics, and where appropriate, MTX (Lederle). Antibiotics were either 100 units/ml penicillin plus 100ug/ml streptomycin (Gibco Bio-cult), or 50ug/ml gentamycin (Schering Corp.).

MTX was obtained either as 25 mg/ml sterile solution, or in powder form. Cultures were usually grown in plastic flasks (Nunc, 25 cm^2, 75cm^2 or 174cm^2); larger scale cultures were grown in horizontally rotating 2½l glass bottles. An atmosphere of 5-10% CO_2 was used and the temperature was 35-37°C.

Routinely, suspension culture cells were diluted about three-fold into fresh meduim every two to four days; anchored cells were released by trypsinization and replated at lower density every two to seven days.

2.3.2. Cloning of Cells

PG19 cells were cloned by plating out at low density, allowing two to five weeks for colonies to grow to the 50-200 cell stage, and then picking individual colonies using stainless steel cloning cylinders. Attempts to clone MTX-resistant EL4 cells using a microwell plate or soft agar were unsuccessful.

2.4. CHROMOSOME ANALYSIS

The standard methods used have been described in ref. 94.

2.5. CELLULAR DNA PREPARATION

DNA was prepared from nuclei in order to exclude mycoplasmal DNA from contaminants present in some of the cultures.

2.5.1. Preparation of nuclei

Nuclei were prepared using a modification of the chromosome isolation method of Maio and Schildkraut (101) Harvested cells were washed twice with Dulbecco's medium, resuspended in 10 volumes of ice-cold 20mM Tris-HCL pH 7.5, 1mM $CaCl_2$, 1mM $MgCl_2$ and left on ice for 20 minutes. This procedure causes the cells to swell. All the following steps were carried out at 0^oC. 5% (v/v) Triton X-100 in the above buffer was added to give a final Triton X-100 concentration of 0.5% (v/v) and the mixture left for a further five minutes to weaken the cell membranes. Cells were then homogenized in a Douce homogenizer with a type B (tight-fitting) pestle until they appeared broken when examined in a microscope under phase contrast. The nuclei were collected by centrifugation at 3,000rpm for five minutes in an MSE Chilspin centrifuge. The pellet was resuspended in 0.5% (v/v) Triton X-100, 20mM Tris-HCl pH 7.5, 1mM $CaCl_2$, 1mM $MgCl_2$, and one of two procedures was adopted. For a crude nuclear preparation the nuclei were resedimented as above. For a clean nuclear preparation the suspension was layered over 4ml of 2.2M sucrose in 20mM Tris-HCL pH 7.5, 1mM $CaCl_2$, 1mM $MgCl_2$, and centrifuged at 30,000rpm in a 10 x 10 Al rotor for one hour. Nuclei formed a pellet at the bottom of the tube while contaminating cells and debris remained in the upper layer or at the interface between the two layers.

2.5.2. Cellular DNA preparations

DNA was prepared by one of two methods. The first was the procedure of Gross-Bellard et al (102) with modifications. Pronase and protease K were used interchangeably. Dialysis against 10mM Tris-HCL pH8, 10mM EDTA, 10mM NaCl was used instead of dialysis against 10mM Tris-HCl pH8, 500mM EDTA, 10mM NaCl since the latter seemed an unnecessary expense and would require subsequent removal of the EDTA before restriction enzyme digestion or electrophoresis could be carried out. A final dialysis step against 1mM Tris-HCl pH8, 0.1mM EDTA was added. Volumes used throughout the procedure were such that the final DNA concentration was $\geq$0.1mg/ml.

The second procedure was that of Ross (103), which involves rapid lysis of cells in a buffer containing 7M urea to reduce degradation of DNA during isolation. DNA was finally dissolved in 1mM Tris-HCl pH8, 0.1mM EDTA. DNA preparations were stored frozen at -20^{o}C or, for periods of a few days, at 4^{o}C.

2.6. ORIGIN, GROWTH AND PREPARATION OF RECOMBINANT PLASMID DNA

2.6.1. Origin of the mouse DHFR cDNA-containing plasmids

Two different plasmids containing mouse DHFR cDNA inserts were used: pDHFR 11 and pDHFR 21. Both were gifts from R.T.Schimke (Stanford University). The construction of pDHFR 21 has been described (104);

a description of the construction of pDHFR 11 has not been published, but it is thought to have been constructed in the same way. pDHFR$^{21}_{\lambda}$ contains a cDNA insert of about 1.5 kilobases (Kb) which corresponds to the 3' nontranslated region and about 90% of the coding region of mouse DHFR mRNA; pDHFR 11 has an insert of about 1.6 Kb corresponding to the 3' nontranslated region, all of the coding region and part of the 5' nontranslated region of mouse DHFR mRNA. The source of the DHFR mRNA used was MTX-resistant S-180 cells (73).

2.6.2. Preparation of plasmid DNA

E coli C600-SR 1592 cells harbouring these plasmids were grown in L broth. pDHFR 21 DNA was prepared by acid-phenol extraction (105) followed by caesium chloride/ethidium bromide density gradient centrifugation (106). pDHFR 11 DNA was a gift from H.J.Cooke.

2.6.3. Origin of other recombinant plasmids and phage

1. pβG1 (107) contains an insert of rabbit β-globin cDNA, and the purified plasmid DNA was a gift from E.M.Southern.
2. pCR1-αG59 contains an insert of rabbit α globin cDNA, and the purified DNA was a gift from N.Hanscombe.
3. pSpG17 contains an insert of sea urchin genomic DNA including part of an actin gene. The plasmid was constructed by Durica and Schloss and purified DNA

was a gift from D.Cooper.

4. λgt WES. rDNA contains a 6.6Kb EcoR1 insert of mouse ribosomal DNA (108).

2.7. DNA ANALYSIS

2.7.1. Restriction enzyme digestion

Restriction enzymes were from several sources. Enzymes were prepared in this laboratory by B.Smith and C.Graham according to standard procedures. In addition, enzymes were purchased from commercial suppliers. Details are given in table 2.1. Digestions were done using conditions recommended by the suppliers. Complete digestion was ensured in various ways. A two to six fold excess of enzyme over that expected to be necessary to digest the DNA in the time allowed was used; in some cases λDNA was added to the reaction as an internal standard, while in other cases λ DNA was added to an aliquot of the reaction mixture but not to the main reaction. Enzymes with recognition sites which include CpG present a special problem because this doublet may be methylated in mouse DNA and many enzymes cleave the methylated DNA slowly or not at all (A.Bird, personal communication). At least a ten-fold excess of these enzymes over that necessary to digest unmethylated DNA was used.

Restriction Enzyme	Source			
	MRC MGU	BRL	B-M	NEB
Acc I	+	.	.	.
Ava I	+	.	.	.
Bam HI	+	+	+	.
Bgl II	+	.	.	.
Eco RI	+	.	.	.
Hae II	+	.	.	.
Hae III	+	+	.	.
Hha I	+	.	.	.
Hind III	+	+	+	.
Hpa I	+	.	.	.
Hpa II	+	.	.	.
Kpn I	.	+	.	.
Msp I	.	.	.	+
Pst I	.	.	+	.
Sma I	+	.	.	.
Tac I	.	+	.	.
Taq I	+	+	.	.
Xho I	.	+	.	.

Table 2.1. Sources of restriction enzymes.

Key: MRC MGU: prepared in this laboratory by B Smith and C Graham.

BRL: purchased from Bethesda Research Laboratories.

B-M: purchased from Boehringer-Mannheim.

NEB: purchased from New England Biolabs.

2.7.2. DNA gel electrophoresis

For most DNA samples, agarose gels 3mm thick, 9, 18, or 36cm long and 18 or 7 cm wide were used. Gel and running buffer was 26mM Na_2HPO_4, 3.3mM NaH_2PO_4, 1mM EDTA; running buffer was recirculated between cathode and anode during electrophoresis. The concentration of agarose varied between 0.25% and 2%, and the voltage gradient between ½V/cm and 10V/cm, depending on the expected size of the DNA fragments of interest. Small DNA fragments were electrophoresed on 0.7mm thick, 18cm long and 17cm wide 6% polyacrylamide gels. Gel and running buffer was 40mM Tris-acetate pH8, 20mM Na acetate, 1mM EDTA and the voltage gradient was 10V/cm.

DNA was loaded in gel buffer containing 3% ficoll to increase the density, and orange G to provide a visible marker so that the rate of migration could be seen.

2.7.3. Gel staining and photography

Gels were stained after electrophoresis by immersing them for about half an hour in approximately 500ml of running buffer to which 1 drop of 10mg/ml ethidum bromide had been added. Occasionally they were then destained by soaking in running buffer without ethidium bromide for half an hour.

For photography gels were transferred to a black perspex plate and illuminated from above with unfiltered ultra-violet emitting lamps (Mineralight). These lamps gave excitation at 254mm and this energetic radiation introduced breaks into the DNA molecules, thus making a depurination step before transfer to nitrocellulose unnecessary. A red filter (Hoya) was placed over the camera lens and the flourescence from the DNA recorded on 5 inch by 4 inch FP4 film (Ilford). After exposure, the film was developed in D76 (Kodak) for about twice the recommended time (10 to 20 minutes depending on the state of exhaustion of the developer and the temperature), and fixed in Amfix (May and Baker).

2.7.4. Analysis of gel photographs

Generally, photographs were scanned using a Joyce-Loebel microdensitometer. In one case a more detailed analysis of EL4 DNA was carried out by Graham Smith of the Royal Observatory, Edinburgh. A 2mm wide strip from the centre part of the photograph of each gel track was scanned by the densitometer COSMOS, and data was stored and manipulated digitally. Unit squares of 16 μm x 16 μm were asigned one of 256 density levels; thus there were 128 measurements of the density for each 16 μm strip across the photograph.

The mode of these densities was determined since a mean would have been strongly influenced by artefacts such as dust particles on the gel or photograph, and

a density versus migration distance profile of the gel tracks was obtained - the raw data from the raw data. A smooth background curve was calculated to fit this "raw data", and was subtracted from the the raw data to give the "background subtracted data". These curves were also compared with one another by subtraction.

2.7.5. Transfer to nitrocellulose

Transfer of DNA to nitrocellulose filters was carried out according to the method of Southern (109). Nitrocellulose was from Millipore, Schleicher and Schull, or Sartorius. Filters were stored at room temperature after baking, often for many months.

2.7.6. Preparation of radioactive probes

DNA was labelled with ^{32}P by nick translation (110), usually using two α-^{32}P deoxyribonucleotide triphosphates (Amersham). The specific activity of the ^{32}P labelled product obtained varied between 10^7 and $3x10^8$ dpm per ug. The most highly radioactive probes did not lead to the highest levels of hybridization, so a specific activity of about 10^8 dpm per ug was commonly used. Total cellular DNA for a "Southern cross" hybridization experiment was labelled by nick translation at endogenous nicks only; DNase 1 was omitted from the reaction mixture. A fragment of pDHFR 21 labelled with ^{125}I by nick translation was a gift from C.J.Bostock. Subfragments of pDHFR 11 used as probes in one gene mapping experiment were

prepared as follows. DNA was digested either with both Pst I and Hae III, or with Bgl II alone, ethanol precipitated and redissolved in distilled water. 500ng of the mixture of restriction fragments was nick translated for 15 minutes, and the entire reaction mixture applied to the top of a 6% polyacrylamide gel. After electrophoresis for two hours, the wet gel was covered with cling film (Snappies snap-wrap), and autoradiographed at room temperature for 10 minutes. The appropriate band was excised from the gel with a scalpel blade, cut into small fragments, and the polyacrylamide-DNA mixture boiled and added to a hybridization mix (see section 2.7.7.).

2.7.7. Prehybridization, hybridization and washing of filters

Filters were wetted in 2SSC and inserted into cylindrical glass tubes about 25cm long and 5cm in diameter. Air bubbles between the filter and the glass were squeezed out and about 20ml of prehybridization mix added. This was 4 to 6 X SSC containing 4 to 6 X Denhardt's solution (111). The tube was sealed and placed horizontally in a rotating aparatus in a 65°C oven. After two to five hours, the prehybridization mix was poured out and replaced by 10ml of hybridization mix. This was 6 x SSC containing 6 x Denhardt's solution, 0.5% SDS, a labelled probe, 50 ug/ml sonicated sea urchin or E coli DNA, 50 ug/ml yeast RNA and sometimes 10 ug/ml poly A and 10 ug/ml poly C.

Probe and competitor nucleic acids were boiled for five minutes before being added. In later experiments 10% dextran sulphate was included (112). Hybridization time in the absence of dextran sulphate was overnight when multiple copy genes were being sought, and over two nights for single copy genes. In the presence of dextran sulphate, a period of three to five hours was generally used; occasionally hybridizations were left overnight.

After hybridization, filters were washed in a flat glass dish containing about 200ml of solution. The standard conditions used for a homologous probe were 0.1 x SSC, 0.1% SDS at 52°C. For a non-homologous probe the SSC concentration was increased to 1 x or 3 x in order to retain poorly matched DNA duplexes. The wash mix was exchanged and the filters blotted dry each 20 minutes until the background on the filter was reduced to an acceptably low level, as judged using a radiation monitor. The time required was usually one to two hours.

2.7.8. Visualization of bands

After washing, the filters were dried in a 65°C oven for 10 minutes, put in X-ray film cassetes and covered by a layer of cling-film. A sheet of X-ray film, usually Fuji Rx but sometimes Kodak XR1 or XH1, was pre-flashed such that it would give a background optical density of about 0.15 after development, and put in contact with the cling film. Ilford fast tungstate

intensifying screens were used. Casettes were stored in a -70°C deep freeze for periods varying from half an hour to one month. Occasionally preflashing and intensifying screens were omitted and exposure was at room temperature. Film was developed in Kodak DX-80 for five to 20 minutes and fixed in Kodak FX-40.

2.7.9. Estimation of DHFR gene number by blot quantitation

pDHFR11 or pDHFR 21 was digested with Pst I and known amounts mixed with cellular DNA digested with Hind III. The mixtures were electrophoresed and transferred to nitrocellulose, and DHFR-containing DNA fragments were detected by hybridization to ^{32}P labelled pDHFR 11 or pDHFR 21 as described in sections 2.7.1. to 2.7.8. The hybridization was quantified in two ways. When the amount of hybridization was low, autoradiographs were scanned using a Joyce-Loebel microdensitometer. When the amount of hybridization was high, regions of the nitrocellulouse bearing bands were cut out, the accuracy of excision checked by autoradiography, and the radioactivity counted in 5ml of toluene-based scintillant (5g PPO, 0.3g POPOP per litre of toluene) in a scintillation counter.

The weight of hybridizing cellular DNA was determined by comparing the amount of radioactivity in cellular DNA bands with the amount in bands produced by the known quantities of plasmid DNA on the same filter. The quantity of plasmid DNA was to be chosen such that it

gave bands of similar intensity to the cellular DNA.

The number of DHFR genes per haploid genome was calculated from the weight of cellular DNA hybridized. The cDNA insert sizes in the plasmids were known to be approximately 1600 base pairs (bp) for pDHFR 11 and 1500bp for pDHFR 21, and the haploid genome size of the mouse was assumed to be 3×10^9bp.

2.7.10 Estimation of DHFR gene number by spot hybridization

DNA samples were adjusted to 60 ug/ml. 50ul aliquots (3ug) were denatured by adding 5ul of 0.5M NaOH, 1.5M NaCl, heated to 65°C for 10 minutes and then neutralized by adding 5ul of 0.5M Tris-HCl pH 5.0, 3M NaCl. A 20 cm by 20cm nitrocellulose sheet, ruled into 1.5cm by 1.5cm squares, was wetted in 2 x SSC, and a DNA sample was spotted onto each square. After rinsing in 2 x SSC, the filter was baked at 80°C in a vacuum oven for two to five hours. Prehybridization, hybridization and washing were as described in section 2.7.6. The washed filter was dried and briefly autoradiographed to asess anomalous background. The 1.5cm by 1.5 cm squares were cut out and the radioactivity counted. Standards consisted of 3ug wild-type DNA mixed with known amounts of pDHFR 11 digested with Pst I.

From the amount of hybridization the number of genes was estimated (section 2.7.9.). This method was only used to determine gene numbers in DNA samples from cells with amplified DHFR genes. The technique is similar to that of Kafatos et al (114) and Velten et al (113).

2.8. RNA PREPARATION

Initially two methods of RNA preparation were tried. The method of Ross (103) gave RNA which was degraded, while the guanidine-HCl method (115) gave very low yields because the guanidinium-nucleic acid precipitate failed to dissolve in the Na_2EDTA at the cation exchange step. Accordingly a combination of the two methods was developed. An unwashed cell pellet was rapidly lysed by adding 9ml 8M guanine-HCl, 20mM Na acetate pH 5, 1mM DDT, or 8M guanine-HCl, 20mM Na acetate pH 5, 10mM mercaptoethanol. The viscous mixture was layered over 2ml 5.7M CsCl, 10mM Tris-HCl pH 8, 1mMEDTA, and centrifuged at 33,000 rpm overnight in an MSE 6 x 14 Ti swing out rotor. RNA formed a transparent pellet at the bottom of the tube. A similar combination of the two methods has been independently developed by Ruddle and coworkers (116).

The RNA pellet was redisolved in 100ul distilled water, a one-tenth volume of 3M Na acetate, 2mM EDTA was added and the RNA was precipitated by addition of two volumes of absolute ethanol. The precipitation step was repeated two more times, and the final RNA pellet was redissolved in distilled water.

2.9. RNA ANALYSIS

Total cellular RNA was run on 20cm long agarose gels containing methyl mercuric hydroxide as a denaturing agent (117). The sample buffer contained 10mM MeHgOH and the gel 5mM MeHgOH. The agarose concentration varied between 0.5 and 2%, and the voltage gradient was between 2 V/cm and 5 V/cm. Ribosomal RNA species, visualized by staining with ethidium bromide, served as internal size markers.

2.9.2. Preparation of DBM paper and RNA transfer

DBM paper was made by the procedure of Alwine et al (118) as modified by Wahl et al (112), using Whatman 540 as the starting material and N-(3-nitrobenzyloxymethyl)-pyridinium chloride from BDH Chemicals Ltd. (batch no. 6858662). Paper was stored as the NBM form and activated just before use. Transfer was carried out by blotting overnight at $4^{o}C$.

2.9.3. Detection of DHFR RNA species

The procedure of Wahl et al (112) was followed. The probe used was ^{32}P labelled pDHFR 21, prehybridization was for two hours in a rotating tube and hybridization was for four hours. Washing and detection of hybrids was as described in sections 2.6.7 and 2.6.8.

2.10.cDNA SYNTHESIS

cDNA was synthesized by A.T.H.Burns using a published protocol (119), with the following modifications. The template was total cellular RNA at a concentration of about 1mg/ml, and the RNase inhibitor was from Searle and was used at 182 units/ml.

2.11.PROTEIN ANALYSIS

2.11.1. Preparation of protein samples

Samples of total cellular protein were prepared for electrophoresis as described previously (94). Briefly, a washed cell pellet was resuspended in distilled water and taken through cycles of freezing and thawing to ensure complete lysis. DNase I was added and the mixture was incubated at 37°C to degrade DNA.

2.11.12. SDS polyacrylamide gel electrophoresis

Gels were run according to the method of Laemmli (120) using a 12% polyacrylamide resolving gel and a 5% polyacrylamide stacking gel. An electrophoresis apparatus was constructed: it consisted of two 20cm by 20cm sheets of window glass separated by 0.7mm thick strips of plastic and connected to the buffer reservoirs at top and bottom. The plastic, purchased from a local toy shop, was a gift from E.M.Southern. The edges were sealed by a trace of vacuumgrease (Edwards) and the apparatus was held together by foldback 1414 clips (Myers). After the resolving

out and equilibrated with 125mM Tris-HCl pH 6.8, 0.1% (w/v) SDS, and then stored frozen at -20°C. Degradation was by Staphylococcus aureus V8 protease (Miles Laboratories) according to the method of Cleveland et al (122). Peptide fragments were separated on a 17% SDS-polyacrylamide gel and visualised by staining with Coomassie blue.

2.11.4. DHFR enzyme activity measurement

DHFR enzyme activity was assayed spectrophotometrically as described previously (94). Cells were grown in medium lacking MTX and samples for DHFR assay were taken every two to three days for two weeks. Measured activity increased for six to eight days and then remained constant in some cell lines and fell in others. If measured activity fell, measurements were extrapolated back to day 0 on the assumption that the decrease was exponential.

CHAPTER 3

RESULTS PART I

3.1. DHFR ENZYME ACTIVITY IS HIGHER IN CRUDE EXTRACTS OF MTX-RESISTANT CELLS THAN IN CRUDE EXTRACTS OF SENSITIVE CELLS.

The specific activity measurements of DHFR in crude extracts of EL4/WILD, LB1 trans/WILD and LS178Y/WILD cells are shown in table 3.1. No L1210/WILD cells were available for assay. DHFR specific activity measurements for MTX-resistant cells are plotted in fig.3.1. as a function of the number of days growth in the absence of MTX. It can be seen that in each case the measured DHFR activity increased for six to eight days as MTX was diluted out of the cells and fresh DHFR was synthesised. Measured activity then fell, at different rates in different cell lines. In order to estimate the activity on day 0, an extrapolation of the data has been made on the assumption that the amount of enzyme throughout the entire period decreased in an exponential fashion as it did after day 8, and that the MTX concentration was negligible after day 8. These estimated values, and the increase over wild-type specific activity, are shown in table 3.1. Also included in this table are the data on PG19 cells (94) and three other MTX-resistant EL4 cell lines (C.J.Bostock, personal communication). It can be seen that, in every MTX-resistant cell line, there is an increase in DHFR specific activity over the parental cell line.

In fig.3.2a. the DHFR specific activity of MTX-resistant cells is shown as a function of the MTX concen--tration, and in fig.3.2b. the increase over wild-type

Cell line	MTX resistance level (M)[1]	Estimated DHFR specific activity[2]	DHFR overproduction
PG19	0	2.8[3]	-
PG19 (S1)	10^{-4}	2 100[3]	750
EL4	0	6.7	-
EL4/3	2.2×10^{-3}	38 000	5 600
EL4/8	1.1×10^{-3}		1 980[4]
EL4/11	1.1×10^{-3}		890[4]
EL4/12	1.1×10^{-3}		1 280[4]
LB1 trans	0	2.0	-
LB1 trans	4.4×10^{-3}	850	425
L1210	10^{-4}	1 000	unknown
L5178Y	0	3.9	-
L5178Y	4.4×10^{-3}	8 000	2 050

Table 3.1. DHFR specific activity estimates in crude extracts of wild-type and MTX-resistant cell lines.

[1]the level of MTX used routinely for maintenence.

[2]in units: 1 unit oxidizes 1 nmole of NADPH/15min/mg. protein

[3]ref 94.

[4]CJ Bostock, personal communication.

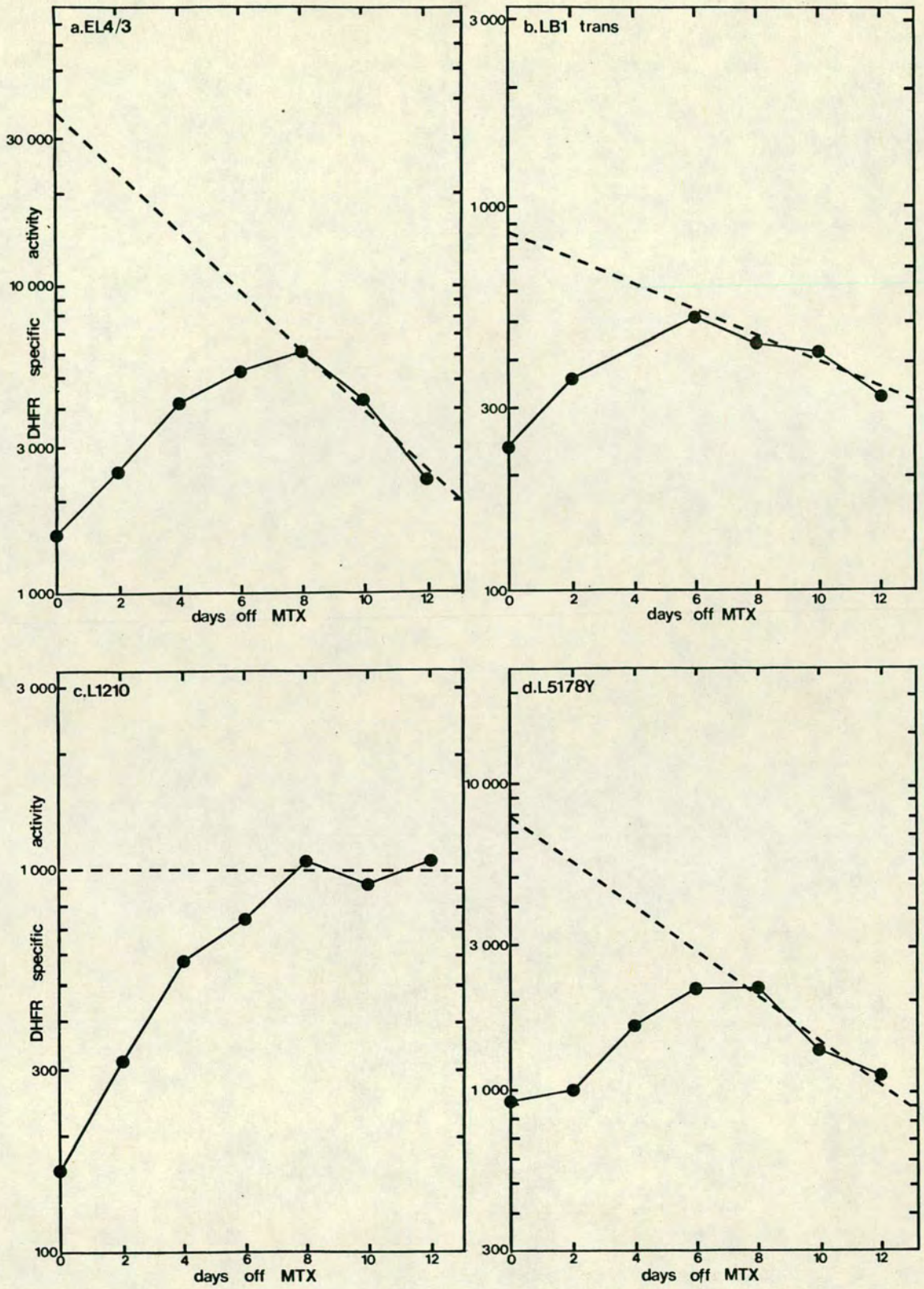


Fig. 3.1. DHFR enzyme specific activity measured in crude extracts of MTX-resistant cells during growth in the absence of MTX.

a. EL4/3:$MTX_R^{2.2\times10^{-3}M}$ cells, b. LB1 trans:$MTX_R^{4.4\times10^{-3}M}$ cells,

c. L1210:$MTX_R^{10^{-4}M}$ cells, d. L5178Y:$MTX_R^{4.4\times10^{-3}M}$ cells.

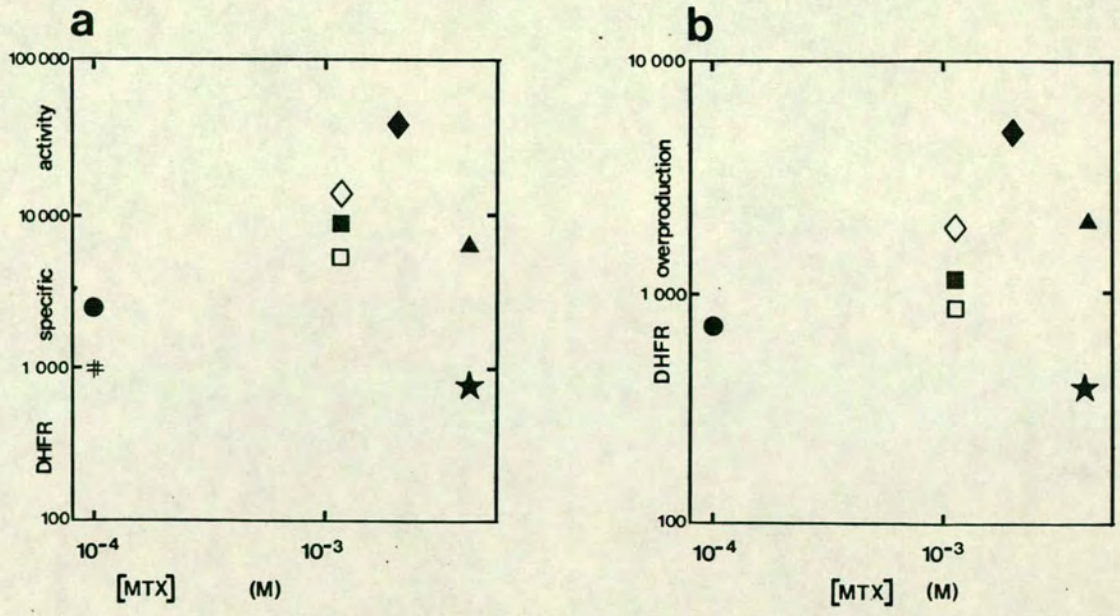


Fig. 3.2. Comparison of DHFR enzyme activity and MTX resistance level in independently selected MTX-resistant cell lines.

a. DHFR specific activity estimated for 'day 0' (y axis) is plotted against the MTX concentration (x axis).

b. DHFR overproduction, the DHFR specific activity estimated in crude extracts of MTX-resistant cells divided by the DHFR specific activity measured in crude extracts of the parental cells, (y axis) is plotted against the MTX concentration (x axis).

- ● PG19T3:$MTX_R^{10^{-4}M}$S1
- ◆ EL4/3:$MTX_R^{2.2x10^{-3}M}$
- ◇ EL4/8:$MTX_R^{1.1x10^{-3}M}$
- □ EL4/11:$MTX_R^{1.1x10^{-3}M}$
- ■ EL4/12:$MTX_R^{1.1x10^{-3}M}$
- ★ LB1 trans:$MTX_R^{4.4x10^{-3}M}$
- # L1210:$MTX_R^{10^{-4}M}$
- ▲ L5178Y:$MTX_R^{4.4x10^{-3}M}$

specific activity (relative specific activity) is shown as a function of the MTX concentration. There is no simple relationship between MTX concentration and either DHFR specific activity or DHFR relative specific activity of the different cell lines. For a discussion, see section 4.2.

3.2. MTX INHIBITION CURVES OF DHFR ACTIVITY IN WILD AND RESISTANT CELLS ARE SIMILAR

DHFR activity in crude extracts of cells has been measured as a function of the concentration of MTX added to the assay mix, and fig.3.3. shows the measured activity expressed as a percentage of the activity measured in the absence of MTX and plotted against MTX concentration. In each case DHFR activity is inhibited by MTX, and the inhibition curve is similar in shape in wild and resistant cells. If a mutant DHFR, which was resistant to MTX inhibition because of decreased MTX binding, had been selected, the inhibition curves of wild and resistant cells would be different. Thus it seems that, in these cells, a structural alteration in the DHFR protein does not contribute to resistance.

The results presented in sections 3.1. and 3.2. lead to the conclusion that a structurally unaltered DHFR has been overproduced in these MTX-resistant cell lines. This is tested in sections 3.3. and 3.4. by examining and characterizing total cellular polypeptides and putative DHFR protein.

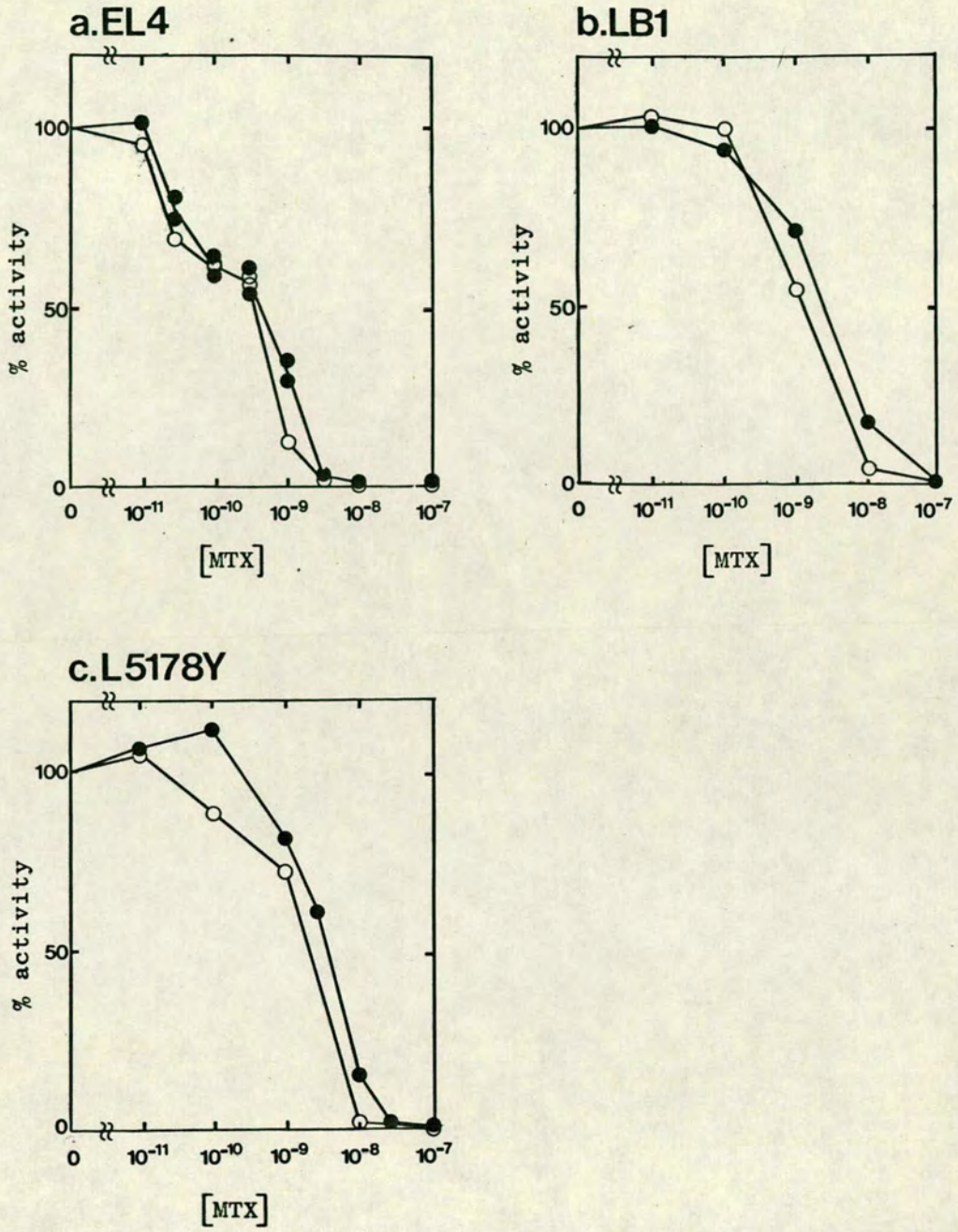


Fig. 3.3. Inhibition of DHFR enzyme activity by MTX in vitro in crude cell extracts. In each of a - c, the concentration of MTX (in M) in the assay mix is shown on the x axis, and the % of the DHFR enzyme activity measured in the absence of MTX is shown on the y axis. —○— = wild type, —●— = MTX-resistant.

3.3. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF TOTAL CELLULAR PROTEIN SHOWS THAT ONE POLYPEPTIDE IS OVER--PRODUCED IN MTX-RESISTANT CELLS.

Washed cells have been lysed, treated with DNase I to degrade cellular DNA, and the proteins analysed by polyacrylamide gel electrophoresis. Fig.3.4. shows the patterns seen when successive stages of the PG19 $S1$ selection were analysed. In the PG19T3:$MTX_R^{3.42x10^{-6}M}S1$ protein it is clearly visible, and it becomes more prominent in protein samples from cells resistant to higher levels of MTX until, in the PG19T3:$MTX_R^{10^{-4}M}S1$ protein, it is the most abundant polypeptide in the cell and accounts for between 3 and 4% of the total cellular protein as judged by quantitation of a scan of the stained gel. This polypeptide migrates at a similar rate to DHFR purified from PG19T3:$MTX_R^{10^{-4}M}S1$ grown as tumours (a gift from M.Mader and C.J.Bostock) and analysed in a parallel track. Purified DHFR and PG19T3:$MTX_R^{10^{-4}M}S1$ cell protein were mixed, and it can be seen that the DHFR and overproduced polypeptide do indeed co-migrate in the mixture, thus providing evidence that the protein that is overproduced in the MTX-resistant cells is DHFR.

Fig.3.5. shows a similar analysis of protein from wild-type and MTX-resistant EL4, LB1 trans, L1210 and L5178Y cells. In each case, an extra band is seen in the polypeptides from MTX-resistant cells which migrates at approximately the same rate as DHFR. The intensity of this extra band, as a proportion of total cellular

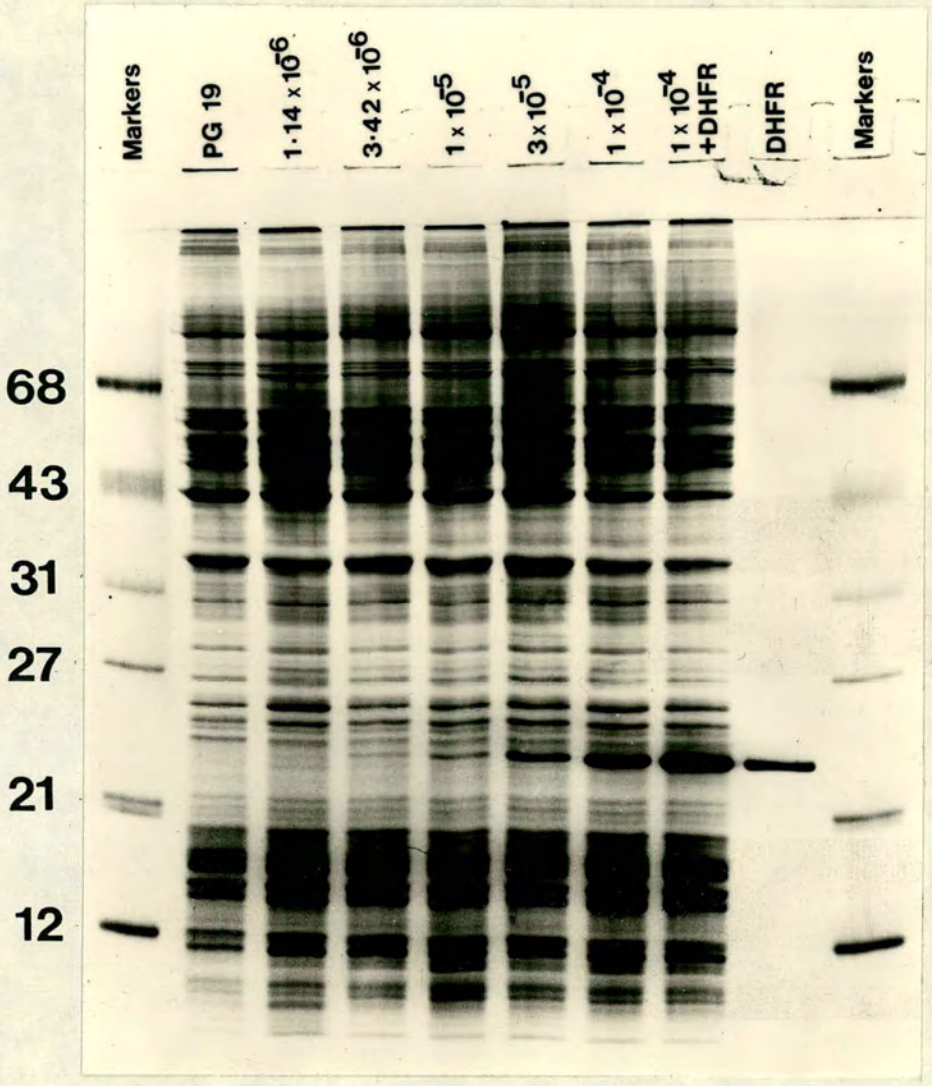


Fig. 3.4. SDS-polyacrylamide gel electrophoresis of total cellular protein from successive stages in the selection of the PG19 S1 cells. The level of MTX used for selection is shown at the top of each track. The sizes of the marker proteins (in kilodaltons) are shown on the left-hand side.

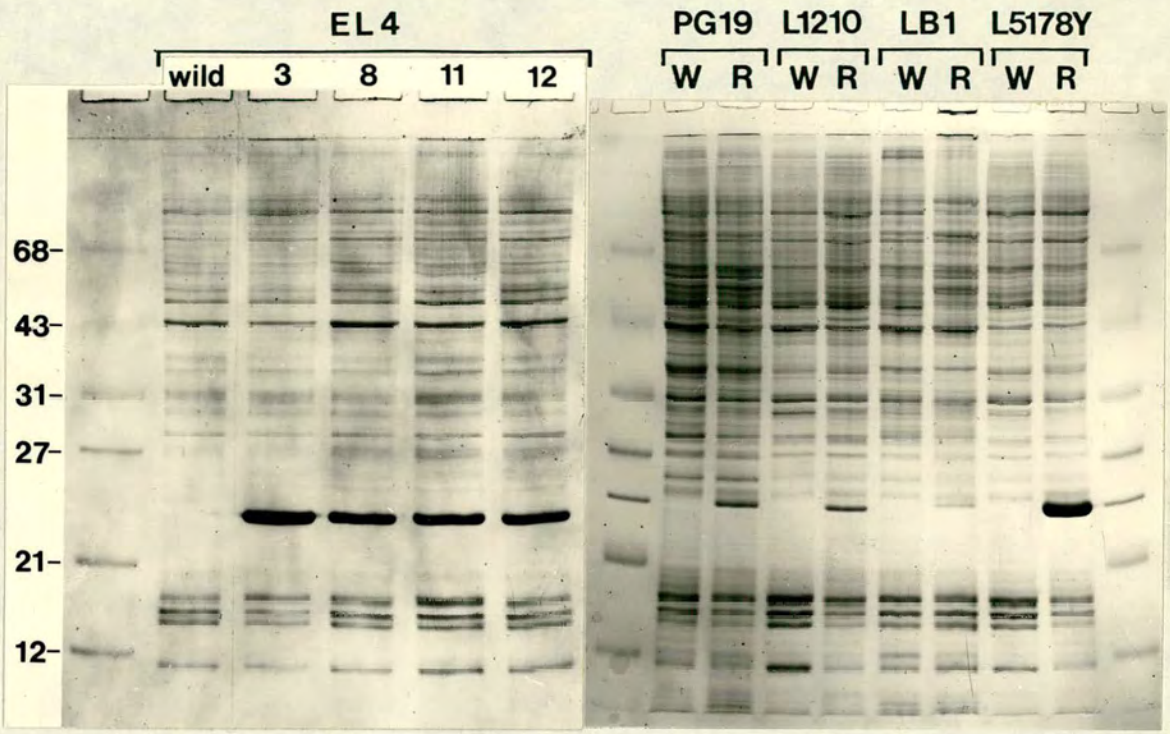


Fig. 3.5. SDS-polyacrylamide gel electrophoresis of total cellular protein from wild-type and MTX-resistant cells. Samples are identified at the top of each track (W = wild-type, R = MTX-resistant). The sizes (in kilodaltons) of the marker proteins are shown on the left-hand side.

protein, does not correlate well with the MTX resistance level. For example, it is only just visible in the LB1 trans:$MTX_R^{4.4x10^{-3}M}$ cell protein although these cells grow at the highest MTX level used, while in the EL4/3:$MTX_R^{2.2x10^{-3}M}$ cell protein, growing at half this MTX concentration, the polypeptide is so abundant that it is barely possible to visualise other cellular proteins without grossly overloading the gel in the DHFR region.

Mixing experiments have shown that the migration rates of purified PG19 DHFR and the overproduced polypeptide in all except one of the resistant cell lines are indistinguishable. The exception is the polypeptide L1210:$MTX_R^{10^{-4}M}$ cell protein, which migrates slightly, but significantly, faster than PG19 DHFR (fig.3.6.)

3.4. PEPTIDE MAPPING BY PARTIAL PROTEOLYSIS SHOWS THAT THE OVERPRODUCED POLYPEPTIDE IS DHFR

Purified PG19 DHFR has been mixed with varying amounts of Staphylococcus aureus V8 protease and the peptide fragments have been analysed by SDS polyacrylamide gel electrophoresis. Fig.3.7a. shows that specific degradation products are observed, and that 0.5 ug of protease generates a suitable series of 10 to 15 bands. Fig.3.7b. shows that degradation occurs to the same extent when the source of the DHFR is a band excised from a polyacrylamide gel instead of the purified protein.

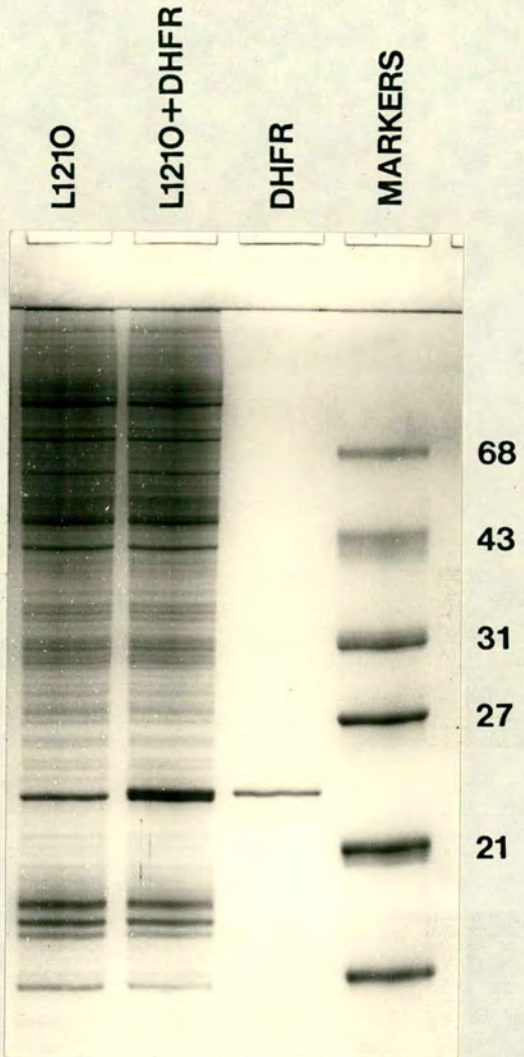


Fig. 3.6. Demonstration of a difference in gel mobility between L1210 DHFR and PG19 DHFR. Total cellular protein from L1210:$MTX_R^{10^{-4}M}$ cells (L1210), or DHFR purified from PG19 cells (DHFR), or a mixture of the two were electrophoresed in a 12½% polyacrylamide gel containing SDS. Note that L1210 DHFR runs slightly faster than PG19 DHFR, and that the DHFR band appears as a doublet in the mixture. The sizes (in kilodaltons) of the marker proteins are shown on the right-hand side.

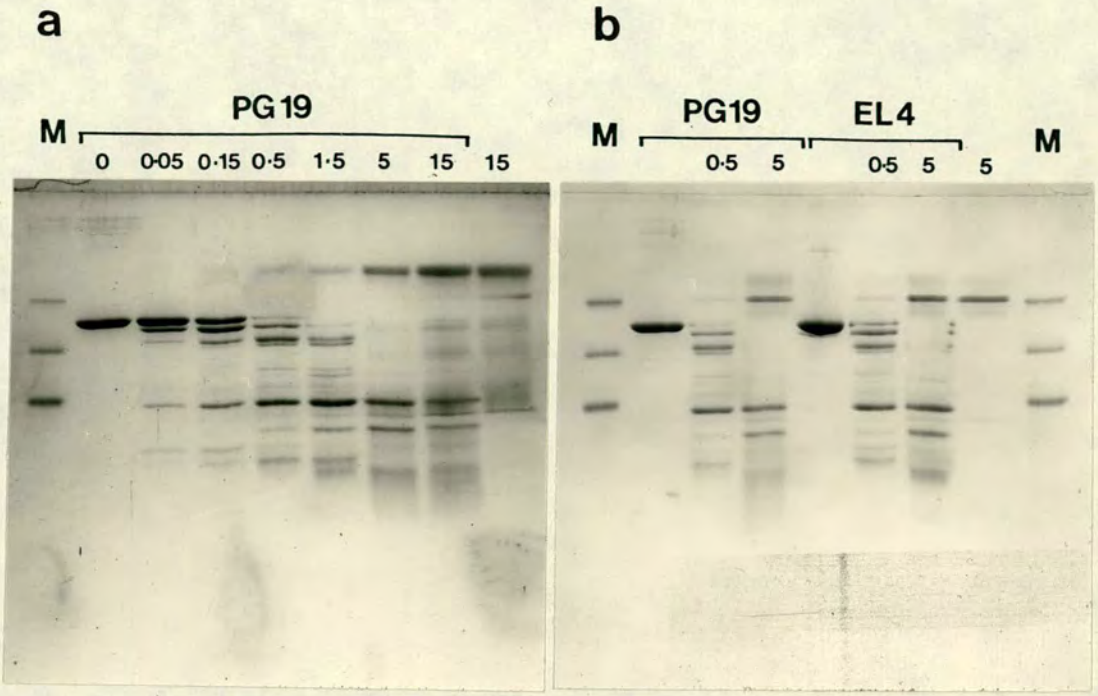


Fig. 3.7. Partial proteolysis of DHFR by Staphylococcus aureus V8 protease.

a. About 10ug of purified PG19 DHFR was mixed with the amount of protease shown at the top of each track and the polypeptides were separated by electrophoresis in a 17½% polyacrylamide gel containing SDS. The right-hand track shows the peptide pattern produced by the highest amount of protease alone.

b. About 10ug of purified PG19 DHFR (PG19) or a gel slice containing putative DHFR from EL4/3:$MTX_R^{2.2x10^{-3}M}$ cells (EL4) was mixed with 0.5ug or 5ug of protease and the polypeptides were separated by electrophoresis in a 17½% polyacrylamide gel containing SDS.

M = marker proteins with sizes (from top to bottom) 27Kd, 21Kd, 12Kd.

The polypeptide overproduced in PG19T3:$MTX_R^{10^{-4}M}$S1 cells, EL4/3:$MTX_R^{2.2x10^{-3}M}$ cells, L1210:$MTX_R^{10^{-4}M}$ cells, and L5178Y:$MTX_R^{4.4x10^{-3}M}$ cells has been excised from a gel and subjected to proteolysis by 0.5 ug S. aureus V8 protease. It can be seen from fig 3.8a. that the degradation products are similar in each case. This shows that:

1. The overproduced polypeptide is similar in amino-acid structure in each cell line
2. it is DHFR.

It seems, therefore, that the small difference in the mobility of the L1210:$MTX_R^{10^{-4}M}$DHFR shown in fig.3.6. is not due to a major alteration in the amino-acid sequence of this DHFR. Such a mobility difference could possibly be caused by a single amino-acid change leading to the polypeptide binding a different number of SDS molecules, or by a difference in a post-translational modification such as addition of carbohydrate residues. This matter has not been pursued.

When protein from LB1 trans:$MTX_R^{4.4x10^{-3}M}$ cells was analysed in the same way, the degradation polypeptides could not be seen. It was thought that this was because the small amount of protein present (fig.3.5.) made location of the band difficult and hence excision of the band inaccurate. Therefore a modification of the method of Cleveland et al (122) was introduced: instead of a single band, entire cellular protein,

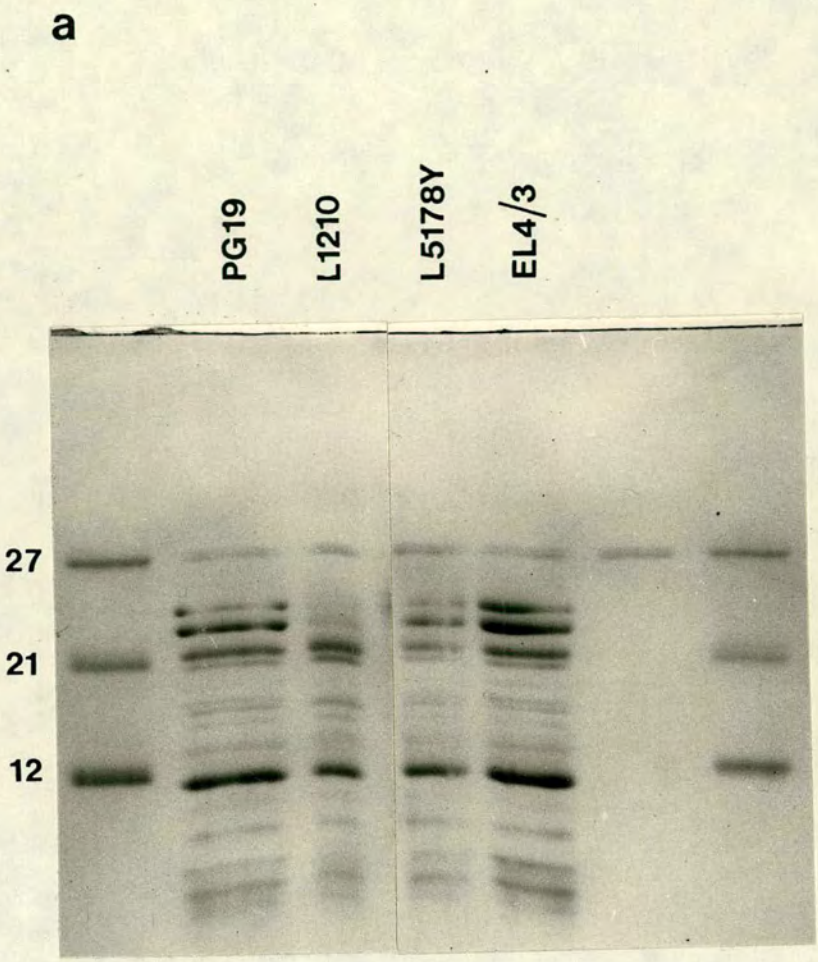


Fig. 3.8. Comparison of the structure of DHFR from different MTX-resistant cell lines using partial proteolysis by Staphylococcus aureus V8 protease.

a. A putative DHFR band was excised from a gel similar to that shown in fig. 3.5. and mixed with 0.5ug protease. The polypeptides were separated by electrophoresis in a 17½% polyacrylamide gel containing SDS. The sizes (in kilodaltons) of the marker proteins are shown on the left-hand side.

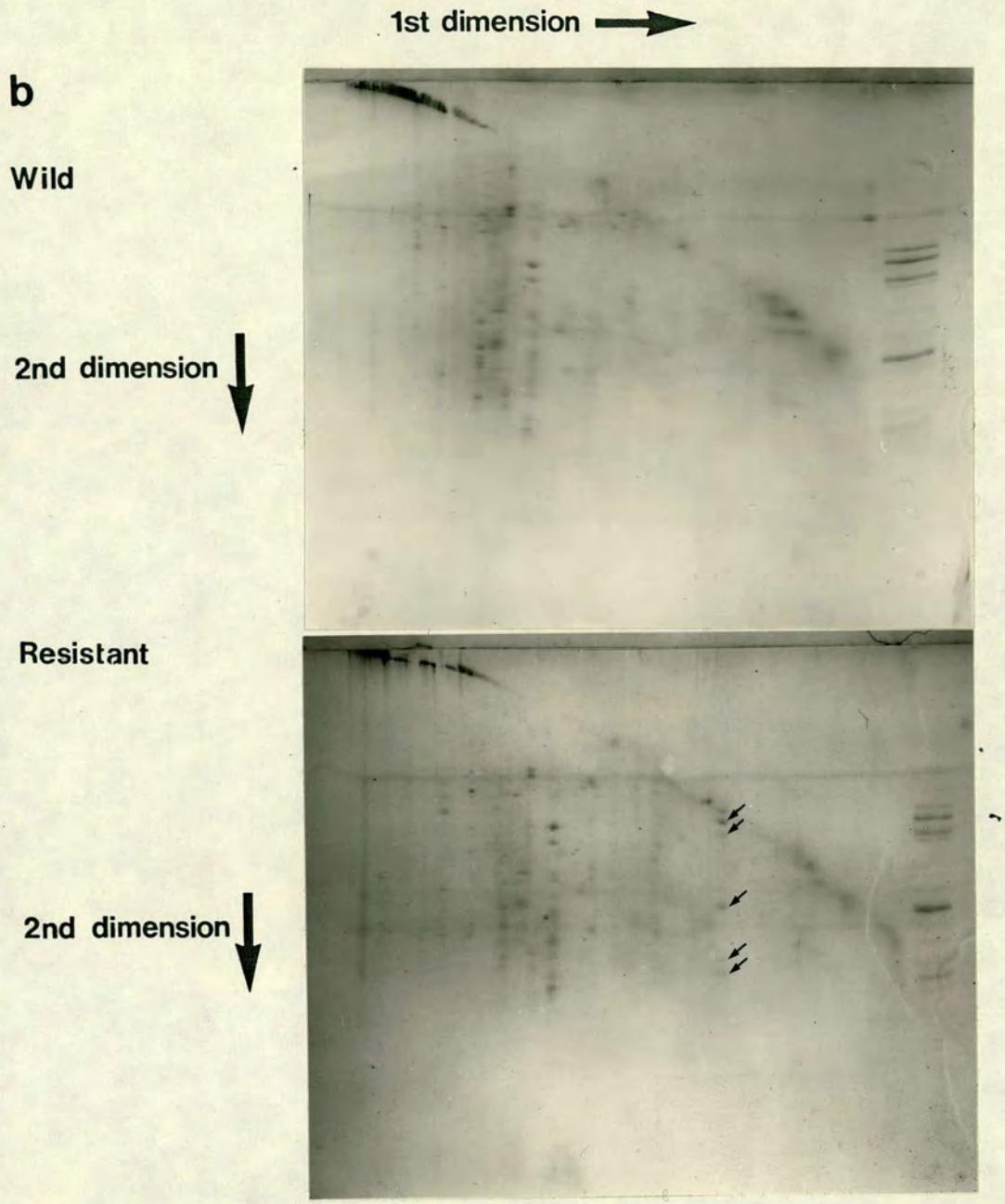


Fig. 3.8. (continued).

b. Total cellular protein from wild-type or MTX-resistant LB1 trans cells was separated in the first dimension by electrophoresis in a 12½% SDS-polyacrylamide gel, as shown in fig. 3.5. A gel strip was then laid along the top of a 17½% SDS-polyacrylamide gel, Staphylococcus aureus V8 protease was added, and electrophoresis was carried out in the second dimension. On the right-hand side of each gel is shown PG19 DHFR partially digested with the same protease. A series of spots which are present in the resistant cell protein but not in the wild-type cell protein and which co-migrate with the major degradation products of PG19 DHFR are arrowed.

separated in one dimension by polyacrylamide gel electrophoresis, was subjected to proteolysis. Fig.3.8b. shows the result of such an experiment. A two-dimensional array of spots is generated. Most of the spots seen when LB1 trans:$MTX_R^{4.4x10^{-3}M}$ cell protein was analysed could also be seen when LB1 trans/WILD protein was analysed using the same method. The exceptions were the series of spots arrowed in fig.3.8b., which were generated only from LB1 trans:$MTX_R^{4.4x10^{-3}M}$ cell protein. These spots co-migrate in the second dimension with the major bands produced by degradation of purified PG19 DHFR. Therefore it can be concluded that the polypeptide overproduced in LB1 trans:$MTX_R^{4.4.x10^{-3}M}$ cells is DHFR.

Thus sections 3.3. and 3.4. confirm the results found in sections 3.1. and 3.2. Together they show that all the MTX-resistant cell lines characterized here overproduce DHFR, and that no extensive alteration of the enzyme has taken place. It seems, however, that factors other than overproduction of DHFR must be contributing to MTX resistance. While one candidate for such a factor is MTX transport, the subject has not been investigated further. The next step was to investigate the basis of DHFR overproduction.

3.5. INCREASED AMOUNTS OF DHFR-SPECIFIC RNA ARE FOUND IN MTX-RESISTANT CELLS

DHFR-specific RNA was examined in sensitive and MTX-resistant cells in order to determine whether DHFR RNA is overproduced in the resistant cells. Total cellular RNA was separated according to size by gel electrophoresis under denaturing conditions and transferred to DBM paper. DHFR-specific RNA was detected by hybridization to ^{32}P labelled pDHFR 21 and autoradiography, and fig.3.9. shows the result. From fig.3.9a. it can be seen that approximately the same amount of RNA has been applied to each track of gel; despite this, hybridization is seen only to RNA from MTX-resistant cells in fig 3.9b., showing that DHFR RNA is increased in amount in these cells and therefore that DHRF enzyme overproduction is associated with DHFR RNA overproduction.

Multiple species of RNA are visible in all resistant cell RNA tracks, and the species are the same size in each track. There are four intense bands smaller than 18S ribosomal RNA, and two feint bands intermediate in size between 18S and 28S rRNA. The origin and biological function of these species cannot be determined from fig.3.9. However, work done by others (123); R.T.Schimke, personal communication) has shown that all the four smaller sized molecules can be found in the polyadenylated fraction of polysomal RNA from mouse liver, and that at least the largest and smallest

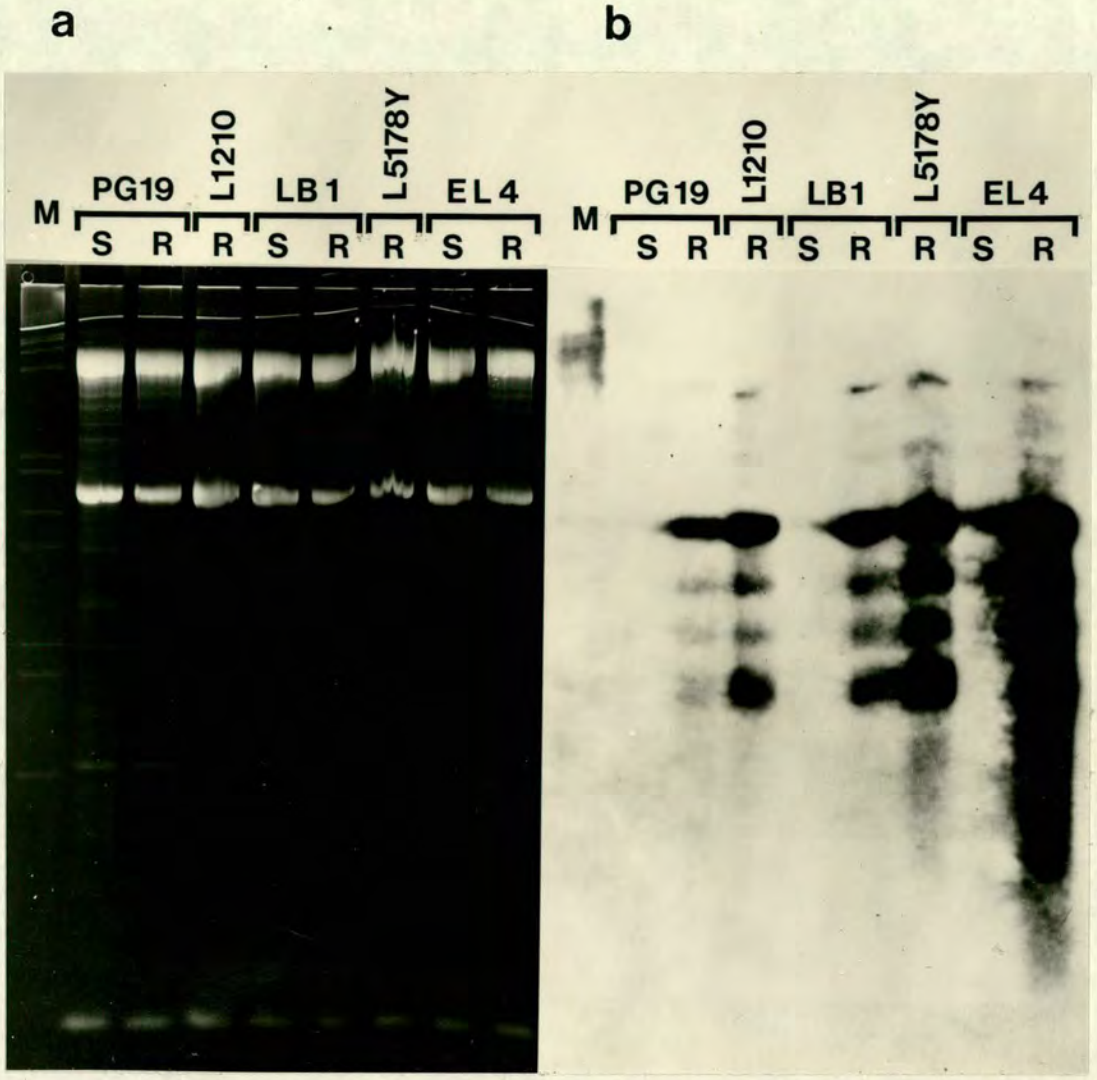


Fig. 3.9. Analysis of total cellular RNA from wild-type and MTX-resistant cells.

a. Aliquots of total cellular RNA were separated by electrophoresis in a 2% agarose gel containing methyl mercuric hydroxide as a denaturing agent. The gel was stained with ethidium bromide, illuminated with u/v lamps, and the fluorescence was photographed. The source of each RNA sample is shown at the top of each gel track (S = sensitive, R = resistant; EL4 R cells were EL4/3. M = marker DNA, which was λ double digested with Eco RI and Hind III). Note that approximately equal amounts of RNA have been loaded onto each track. The prominant RNA species are, from top to bottom, 28S rRNA, 18S rRNA and 4S RNA.

b. The RNA shown in a was transfered to DBM-paper, hybridized with ^{32}P-labelled pDHFR 21 and autoradiographed. Shorter exposures of the EL4/R track showed the band pattern seen in the other R tracks.

of these four are active in an *in vitro* protein synthesizing system. Thus it appears that the multiple species of DHFR mRNA are naturally found in the mouse, and are not a feature peculiar either to *in vitro* cultured tumour cells or to MTX-resistant cells. Since these RNA species are presumably translated in mouse liver, the increased amounts in the MTX-resistant cells are likely to be translated to give increased amounts of DHFR. The nature of the two larger species is unknown, but it is possible that they are precursor froms of the mature mRNA.

Taken together, this evidence suggests that DHFR overproduction in the cells described here is mediated by increased levels of a structurally unaltered mRNA.

3.6. DHFR GENES HAVE BEEN AMPLIFIED IN MTX-RESISTANT CELLS

The DHFR gene numbers in MTX-resistant cells have been estimated using the methods described in chapter 2. Fig.3.10a. shows the standard curve obtained in a spot hybridization experiment when nick-translated pDHFR 11 was hybridized to EL4/ WILD DNA to which 0-2.25 ng pDHFR 11 had been added. It can be seen that within this range there was a linear relationship between the amount of pDHFR 11 applied to the filter and the amount of ^{32}P-labelled pDHFR 11 which hybridized. The amount of hybridization observed when 3 ug of DNA from MTX-resistant EL4 cells was applied to the same filter fell within this range, and hence the amount of DNA complementary

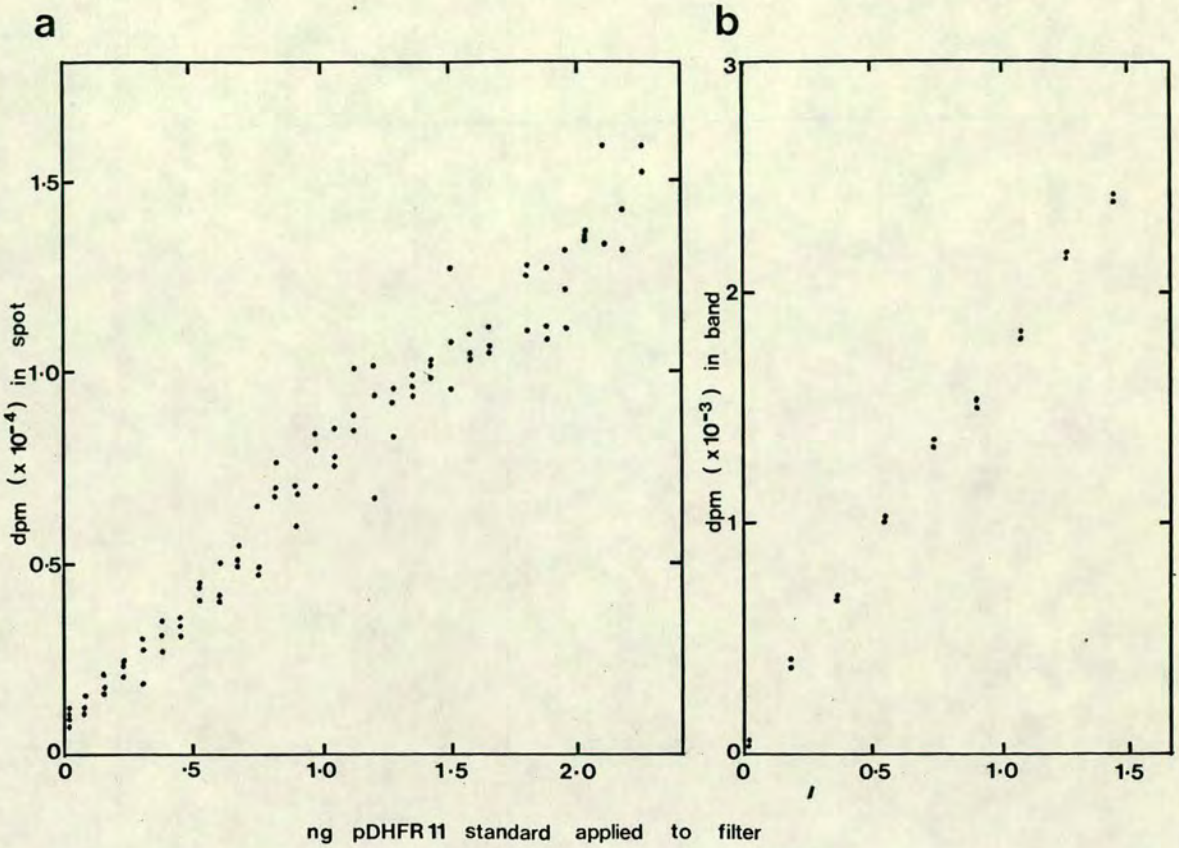


Fig. 3.10. Examples of the standard curves used in the estimation of DHFR gene numbers.

a. Spot hybridization. 0 to 2.25 ng of pDHFR 11 DNA digested with Pst I was mixed with 3 ug of EL4/WILD DNA, denatured, neutralized and spotted onto a nitrocellulose filter. After hybridization with ^{32}P-labelled pDHFR 11 and washing, the radioactivity in each spot was counted in a scintillation counter. The amount of pDHFR 11 standard applied to the filter is shown on the x axis, and the resulting radioactivity is shown on the y axis.

b. Blot quantitation. 0.18 to 1.44 ng of pDHFR 11 DNA digested with Pst I was mixed with cell DNA digested with Hind III, and the mixture was electrophoresed in an agarose gel. The DNA was transfered to nitrocellulose and the filter was hybridized with ^{32}P-labelled pDHFR 11. Bands were located by autoradiography, cut out, and the radioactivity in each was counted in a scintillation counter. The amount of pDHFR 11 standard in each band is shown on the x axis, and the resulting radioactivity is shown on the y axis. The '0' value shown in this experiment is the background radioactivity on a part of the filter lacking DNA.

to pDHFR 11 in the 3 ug EL4 samples could be estimated. These estimates, converted into estimates of the number of DHFR genes per 3 x 10^6Kb of DNA, are included in table 3.2.

Fig 3.10b. shows the standard curve obtained in one experiment when blot quantitation was used to estimate DHFR gene numbers. Again it can be seen that, over the range used, there was a linear relationship between the amount of pDHFR 11 applied to the filter and the amount of ^{32}P-labelled pDHFR 11 which hybridized. Thus, again, the amount of pDHFR 11-complementary DNA in cellular DNA samples on the same filter could be estimated, and the estimates of DHFR gene numbers included in table 3.2. were calculated.

While the total amount of hybridization on different filters varied considerably, because of different specific activities of the probe, different sizes of probe DNA fragments, and different times of hybridization, a linear relationship between the amount of plasmid DNA applied and the amount of hybridization was always found on a given filter. Thus pDHFR 11 acts as an internal standard. The results from a number of such experiments have been combined to give the data in table 3.2.

It can be seen that the estimated number of DHFR genes per 3 x 10^6 Kb of DNA varies from less than a hundred to over a thousand in different MTX-resistant cell lines. The number of DHFR genes in 3 x 10^6Kb of

Cell line	MTX resistance level (M)	Estimated number of DHFR genes per $3x10^6$ Kb of DNA	
		Spot hybridization	Blot quantitation
PG19 (S1)	10^{-4}	.	90
EL4/3	$2.2x10^{-3}$	1 120	750
EL4/8	$1.1x10^{-3}$	1 330	690
EL4/11	$1.1x10^{-3}$	825	600
EL4/12	$1.1x10^{-3}$	1 360	650
LB1 trans	$4.4x10^{-3}$	.	150
L1210	10^{-4}	.	70
L5178Y	$4.4x10^{-3}$	.	200

Table 3.2. Estimates of number of DHFR genes per $3x10^6$ Kb of DNA in independently selected MTX-resistant cell lines.

ld-type DNA is difficult to estimate precicely. Spot ybridization is not a suitable method to use because f the wide variation between different measurements of the same value (fig.3.10a.). Blot quantitation is more precice for two major reasons. Firstly blot quantitation concentrates the DHFR gene sequences: total DNA was typically spread over 15-17cm by gel electrophoresis while the DHFR-hybridizing bands on the filter were each 1-2mm in width; thus there was an approximately 30-50 fold enrichment of DHFR sequences in those regions of the filter contributing to the estimate of DHFR gene number. Secondly, and probably more importantly, the geometry of a blot enables hybridization to DHFR bands to be distinguished from background hybridization, since each band was a narrow rectangle while the background consisted of irregular spots and blotches. This meant that those filters with low backgrounds could be selected for estimation of DHFR gene number; while in a spot hybridization experiment, signal could not easily be distinguished from background and consequently experiments where the background was low could not be chosen. Blot quantitation shows that mouse liver and wild-type cell DNA preparations have about the same number of DHFR genes and that this number is one to two (fig. 3.11a. and fig.3.12.)

Whatever the precise number of DHFR genes in wild-type DNA, and hence whatever the precise degree of amplification, it is clear that extensive amplification has taken place and that it is a major

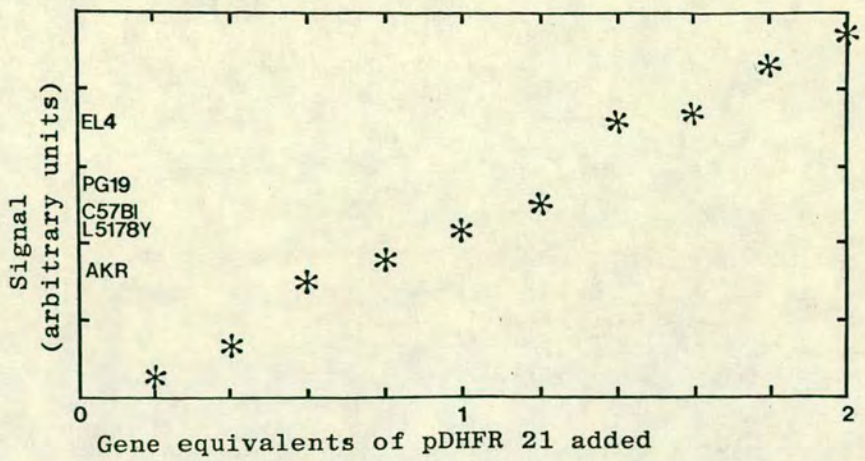


Fig. 3.11.a. Estimation of the number of DHFR genes in mouse liver and wild-type cell line DNA. 10ug of nuclear DNA (cell lines) or total cellular DNA (liver) was digested with Hind III and mixed with various amounts of pDHFR 21 digested with Pst I. The mixture was electrophoresed in a 0.75% agarose gel and DNA was transfered to nitrocellulose. The filter was hybridized with ^{32}P-labelled pDHFR 21 and autoradiographed. The autoradiograph was scanned using a Joyce-Loebel microdensitometer and the areas of peaks were quantitated by cutting out and weighing the paper. The weight of paper (the signal), in arbitrary units, is plotted against the gene equivalents of standard pDHFR 21 added. In addition, the summed weights of the three peaks from each cellular DNA sample are indicated.

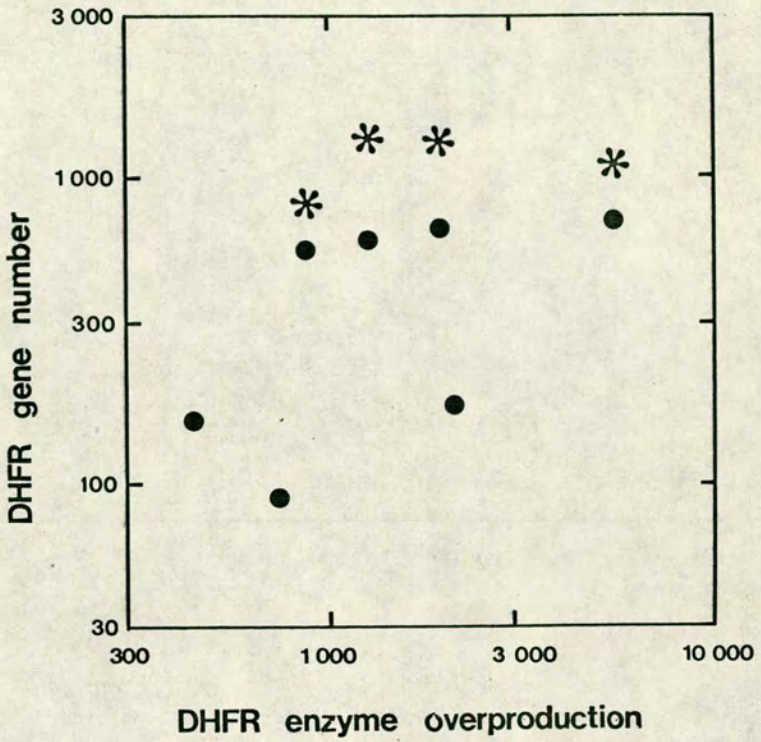


Fig. 3.11.b. Comparison of DHFR gene number and DHFR enzyme overproduction in independently selected MTX-resistant cell lines. DHFR gene number estimates from blot quantitation (●) or spot hybridization (*) experiments are taken from table 3.2. DHFR enzyme overproduction estimates are taken from table 3.1.

factor associated with DHFR enzyme overproduction. Fig 3.11b. shows the estimated number of DHFR genes per 3 x 10^6 Kb of DNA plotted against the degree of DHFR overproduction. It can be seen from the wide scatter of points that there is no simple relationship between the two, suggesting that factors other than gene number can influence the level of DHFR enzyme production. This subject is discussed further in section 4.3.

3.7. DNA BLOTS SHOW THAT UNALTERED DHFR GENES ARE PRESENT IN MTX-RESISTANT CELL DNA, BUT ALSO REVEAL SEVERAL NOVEL FEATURES

The structure of the wild-type DNA gene has been characterized using the Southern blotting procedure. The patterns of restriction fragments of wild-type DNA which hybridize to pDHFR 21 have been determined. Fig 3.12a-c. shows the results obtained from the liver DNA of two inbred mouse strains, PG19T3/WILD, LB1 trans/ WILD, and L5178Y/WILD cells when Bam H1, Eco RI and Pst I were used. It can be seen that each enzyme produced the same pattern in all the DNA samples analysed, and that in each case the patterns consisted of multiple bands. The similarity of the patterns produced in different DNA samples by the same enzyme shows that the structure of the DHFR gene is the same in each sample, at least in the distribution of restriction sites. The multiple bands observed in genomic DNA cannot be generated by

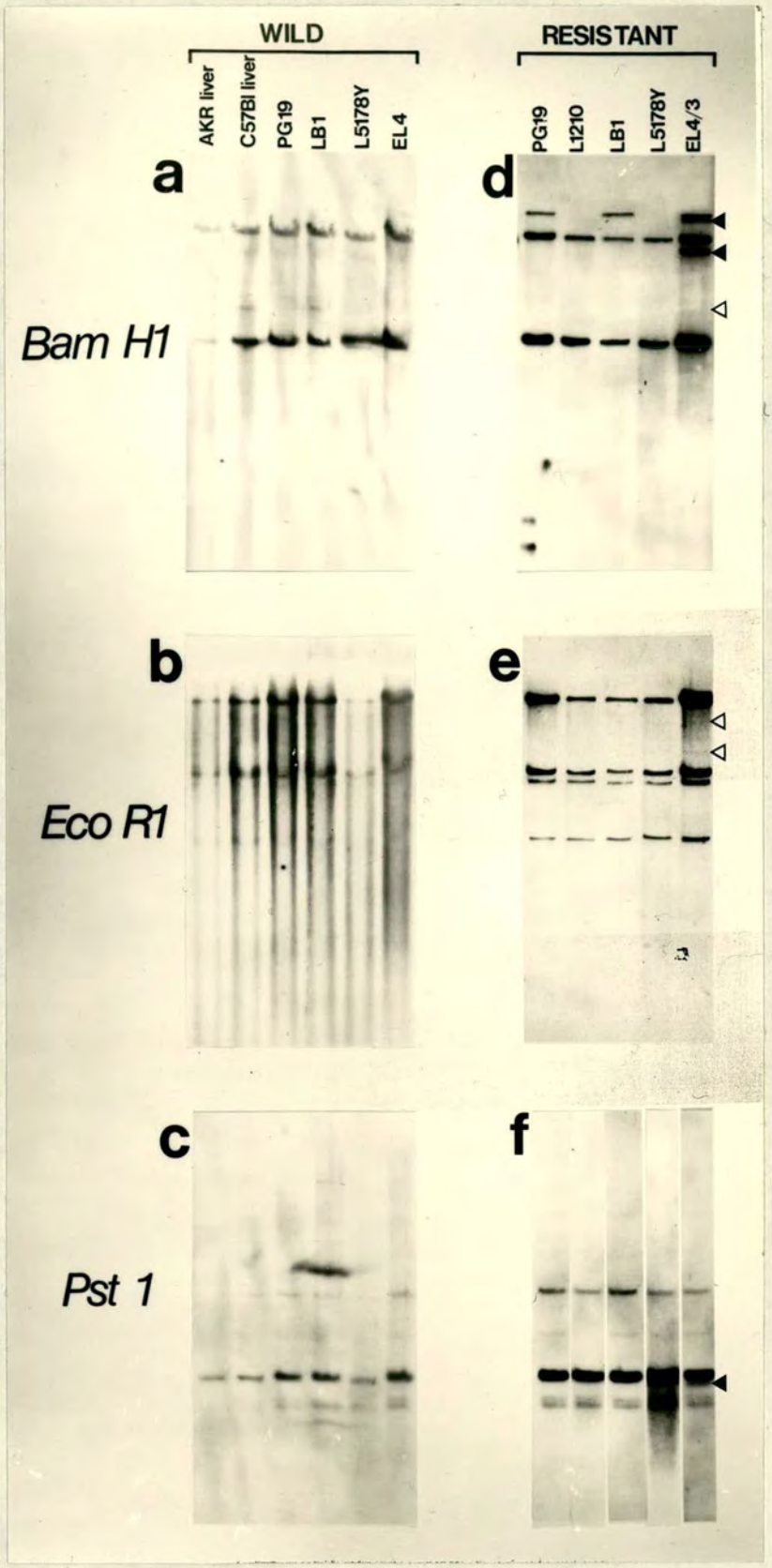
Fig. 3.12. Analysis of the DHFR band patterns in wild-type and MTX-resistant cell DNA.

Mouse liver or wild-type cell line DNA (a,b,c), or MTX-resistant cell line DNA (d,e,f), was digested with Bam HI (a,d), Eco RI (b,e) or Pst I (c,f) and electrophoresed in a 0.75% agarose gel. DNA was transfered to nitrocellulose and the filter was hybridized with ^{32}P-labelled pDHFR 21 and autoradiographed. In wild-type cell DNA samples, the different intensities in different samples of bands of the same size is largely due to different amounts of DNA. In MTX-resistant cell DNA samples, different autoradiographic exposures giving approximately the same band intensities have been combined in the figure.

a,d. The highest molecular weight Bam HI band (58 Kb), visible in three of the MTX-resistant cell DNA samples is believed to be present in all samples but is not seen in other samples because of the small amount of hybridization of pDHFR 21 to it and its susceptibility (because of its large size) to non-specific degradation. Two bands seen only in EL4/3 DNA samples are arrowed (▶) and the positions of some feint bands which appear in suitable exposures of all tracks (see figs. 3.15. and 3.16.) are marked (▷).

b,e. The positions of feint bands which appear in suitable exposures of all tracks are marked (▷).

c,f. A band seen only in EL4/3 DNA is arrowed (▶). Some very feint high molecular weight bands in c are thought to be partial digestion products.


WILD
RESISTANT
AKR liver
C57Bl liver
PG19
LB1
L5178Y
EL4
PG19
L1210
LB1
L5178Y
EL4/3
a
d
Bam H1
b
e
Eco R1
c
f
Pst 1

cleavage within the sequences represented in mRNA since the cDNA lacks sites for the three restriction enzymes used. Two explanations for the multiple bands can be suggested. The mouse could have several different, but cross-reacting, DHFR genes; alternatively there could be a single gene interrupted by introns which contain the restriction sites detected. Since subfragments of pDHFR 11 hybridize to different fragments of genomic DNA (section 3.10.) showing that no genomic fragment contains the entire coding sequence, the latter is likely to be the correct explanation.

The structure of the DHFR gene in DNA from MTX-resistant cells has been characterised in the same wa y, and the results are shown in fig.3.12d-f. It can be seen that all the bands visible in wild-type DNA are also visible here. In addition, other bands can be seen. These can be divided into two categories on the basis of their distribution between different DNA samples:

1. Extra bands which are seen in suitable exposures of all MTX-resistant cell lines,
2. Extra bands which are seen only in a single cell line. EL4/3:$MTX_R^{2.2\times10^{-3}M}$ is the only cell line shown in fig.3.12. which has category 2 bands. They are indicated by solid arrows.

Category 1 bands are thought to be of two types:

1.A. Bands which are thought to be present in wild-types as well as all MTX-resistant cell DNA samples but which, because of technical limitations of

sensitivity, can only be seen when multiple copies of DHFR gene are present. Bands containing only sequences complementary to the 5' end of the DHFR mRNA fall into this subcategory; they are difficult to detect because the probes used lack subsequences from this region (section2.6.1.) and so only a small amount of hybridization resulting in feint bands is possible.

1.B. Bands which are thought to be generated by endogenous DNase cleavage at hypersensitive sites within the DHFR chromatin. Category 2 bands are thought to result from DNA rearrangement. The evidence on which these categories are based is given in sections 3.9. and 3.10. Before considering it, however, another phenomenon revealed by DNA blots will be presented.

3.8. THE DHFR GENE IS EXTENSIVELY METHYLATED AT CpG SEQUENCES

It was found that many of the restriction enzymes used to digest cellular DNA did not produce a pattern of discrete bands as expected, but, even after complete digestion as judged by cleavage of internal λDNA samples, produced a smear which must be comprised of many different fragment sizes that are not resolved by the gel. Examples of such patterns are shown in fig.3.13. The enzymes Sma I and Ava I have been used and it can be seen that the overall patterns produced in both sensitive and resistant DNA are similar, although close inspection reveals many

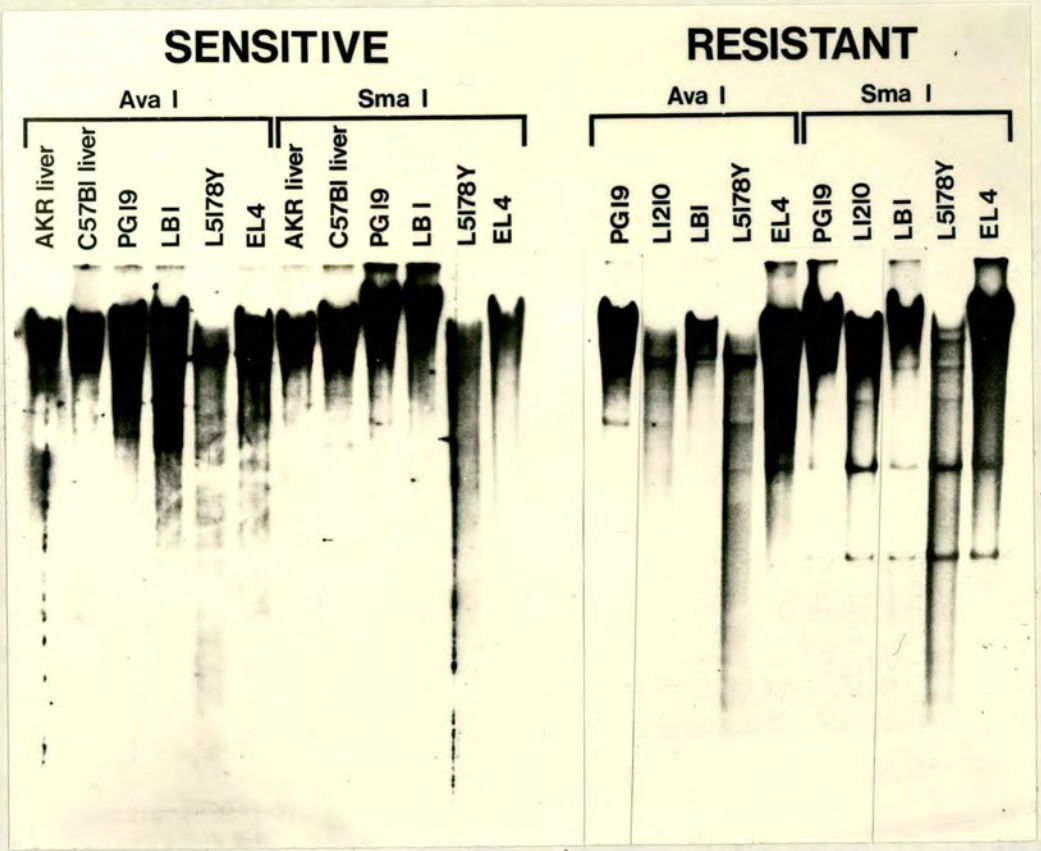


Fig 3.13. Analysis of DHFR genes in sensitive and MTX-resistant cell DNA cleaved with Ava I or Sma I. DNA was digested to completion with the methyl-sensitive enzymes Ava I or Sma I and electrophoresed in a 0.75% agarose gel. After transfer to nitrocellulose, the filters were hybridized with ^{32}P-labelled pDHFR 21 and autoradiographed. Note that in every case a smear of hybridization is seen extending from the top of the gel downwards, and in some cases a few discrete bands are superimposed upon it. The DNA samples are identified at the top of each track.

minor differences. Table 3.3. lists the enzymes which have been found to produce such patterns. Several explanations for these patterns can be suggested.

1. Digestion may be inhibited or absent because of unsuitable conditions in the digestion mix or a poor enzyme preparation.
2. Cleavage sites in the DNA may be absent or blocked.
3. There may be extensive DNA sequence heterogeneity within a cell or between the cells in the population, so that even complete cleavage would not produce a simple pattern of discrete bands.

Explanation 1 can be eliminated because λDNA was used as an internal standard in these reactions and was found to be adequately digested. Explanation 3 can also be eliminated because, as shown in section 3.7., the DHFR gene structure revealed by other restriction enzymes is homogeneous (MTX-resistant EL4 cells form a partial exception: see section 3.10.). Explanation 2 is therefore the most likely. Since the size of the DHFR gene is large (123,124), it is expected on the basis of chance that many sites for most of the enzymes listed in table 3.3. will occur. Also, since part of the cDNA sequence is known (123), the presence of some sites can be predicted. However, it is possible that sites for infrequently-cutting sites such as Tac I are absent. If sites are present but blocked, DNA modification is likely to be the reason. The major form of modification of DNA found in mouse cells is methylation of C residues in CpG doublets to form 5-methyl-CpG. All of the enzymes listed in table 3.3. contain

Restriction enzyme	Recognition sequence	wild-type DNA	MTX-resistant cell DNA
Ava I	CPyCGPuG	+	+
Hae II	PuGCGCPy	+	+
Hha I	GCGC	+	+
Hpa II	CCGG	+	+
Sal I	GTCGAC	.	+
Sma I	CCCGGG	+	+
Tac I	CGCG	.	+

Table 3.3. Restriction enzymes which fail to generate discrete bands after complete digestion of DNA samples and visualization of the DHFR gene sequences.

Key: + = a smear generated, comparable to those seen in fig. 3.13.

. = not determined.

CpG doublets in their recognition sequence. Some have been shown to be incapable of cleavage when their sites are methylated; for others there has been no formal demonstration of methyl-sensitivity, but the possibility exists.

In one case it has been possible to distinguish experimentally between the absence of sites and the presence of methylated sites. The patterns produced by cleavage at CCGG sites by the isoschizomers Msp I and Hpa II have been compared. Msp I cleaves both CCGG and C5mCGG (125); Hpa II cleaves only CCGG (126). Fig 3.14. shows that many CCGG sites are in fact present in the vicinity of the DHFR gene. It should be noted that the Msp I patterns themselves differ in the different cell lines and are also more complex than those shown in fig.3.12. This could be due to a small but variable amount of methylation in the first C of the CCGG recognition sequence: Msp I cannot cleave the sequence 5mCCGG (127). Such methylation is rare but not unknown in mammals (127).

It can be seen from fig. s 3.13. and 3.14. that although there is not extensive cleavage of the DHFR gene by methyl-sensitive enzymes, feint bands can be detected (arrows). Each band must be produced by cleavage at a pair of sites in or near the DHFR gene. Endogenous DNase activity could contribute to this pattern to the extent of cleaving at one or the other end of the fragment (see next section), but cannot be the sole cause by cleaving at both ends, because different patterns are seen when the same DNA preparation is analysed using

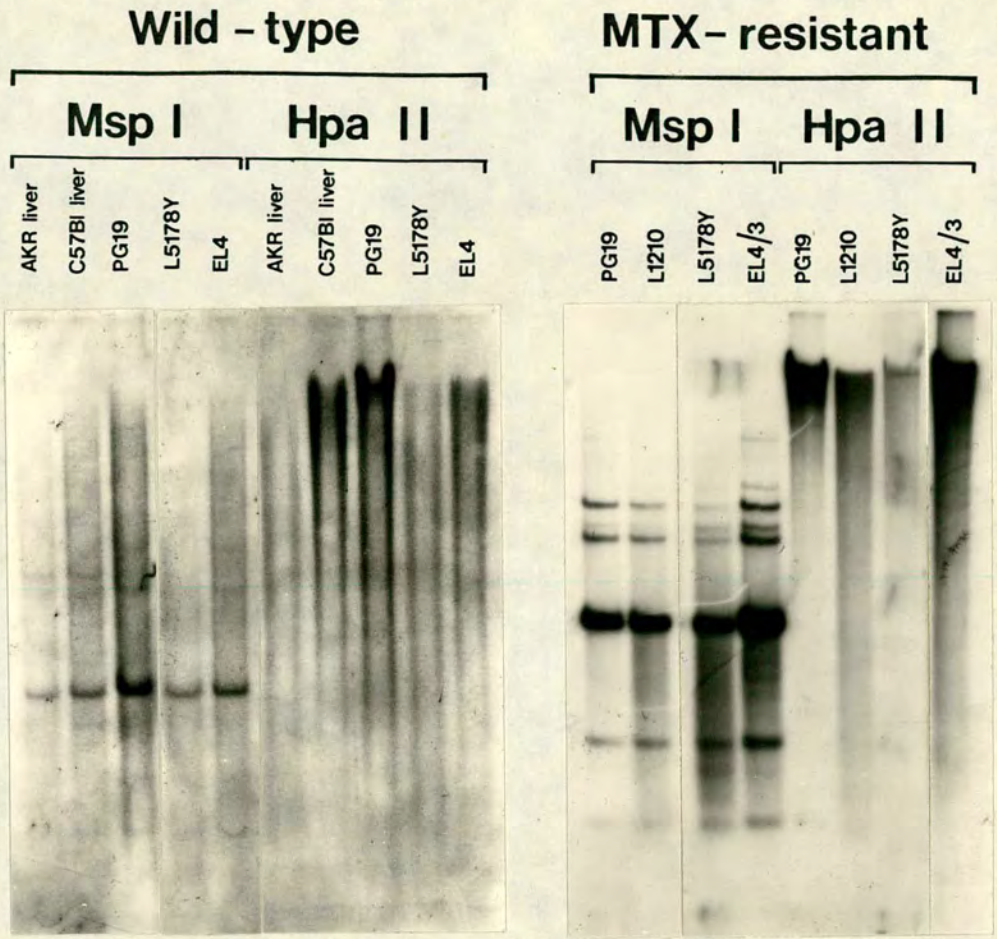


Fig. 3.14. Comparison of DHFR gene sequences after digestion with either Msp I or Hpa II. DNA samples, identified at the top of each track, were digested to completion with either Msp I or Hpa II, as indicated. DNA was electrophoresed in a 0.75% agarose gel (wild-type samples) or a 1% agarose gel (MTX-resistant samples) and transfered to nitrocellulose. The filters were then hybridized with ^{32}P-labelled pDHFR 21 and autoradiographed. The different amounts of hybridization to different wild-type DNA samples are due to different amounts of DNA loaded onto the gel.

different restriction enzymes, and no bands are seen when no restriction enzyme is used. Therefore there must be sites which are unmethylated in a proportion of the DNA molecules.

3.9. SITES HYPERSENSITIVE TO CLEAVAGE BY ENDOGENOUS DNASE ARE FOUND WITHIN AND NEAR THE DHFR GENE.

The feint bands common to all or most MTX-resistant cell DNA samples seen in 3.12d-f. must represent sequences which have been amplified in the MTX-resistant cells, since such bands are not seen in wild-type DNA samples. They have been detected using pDHFR 21, and could therefore be sequences homologous to any portion of the probe: pBR322, the G-C linkers, or the DHFR cDNA insert. These possibilities have been distinguished by using as a sub--probe the DHFR cDNA insert + linkers excised from the complete plasmid by Pst I digestion and purified by gel electrophoresis, and by carrying out the hybridization in the presence of a vast excess of poly C. Fig 3.15. shows that the hybridization patterns obtained under these conditions are similar to those obtained using complete pDHFR 21 as probe. Therefore all of these bands contain sequences complementary to the DHFR portion of the probe.

The experiment shown in fig. 3.15.a. was designed to investigate the degree of homology between these feint bands and DHFR. Two extremes are possible: that the bands have a high degree of homology to DHFR but are present in only a few copies per haploid genome, or that they have a

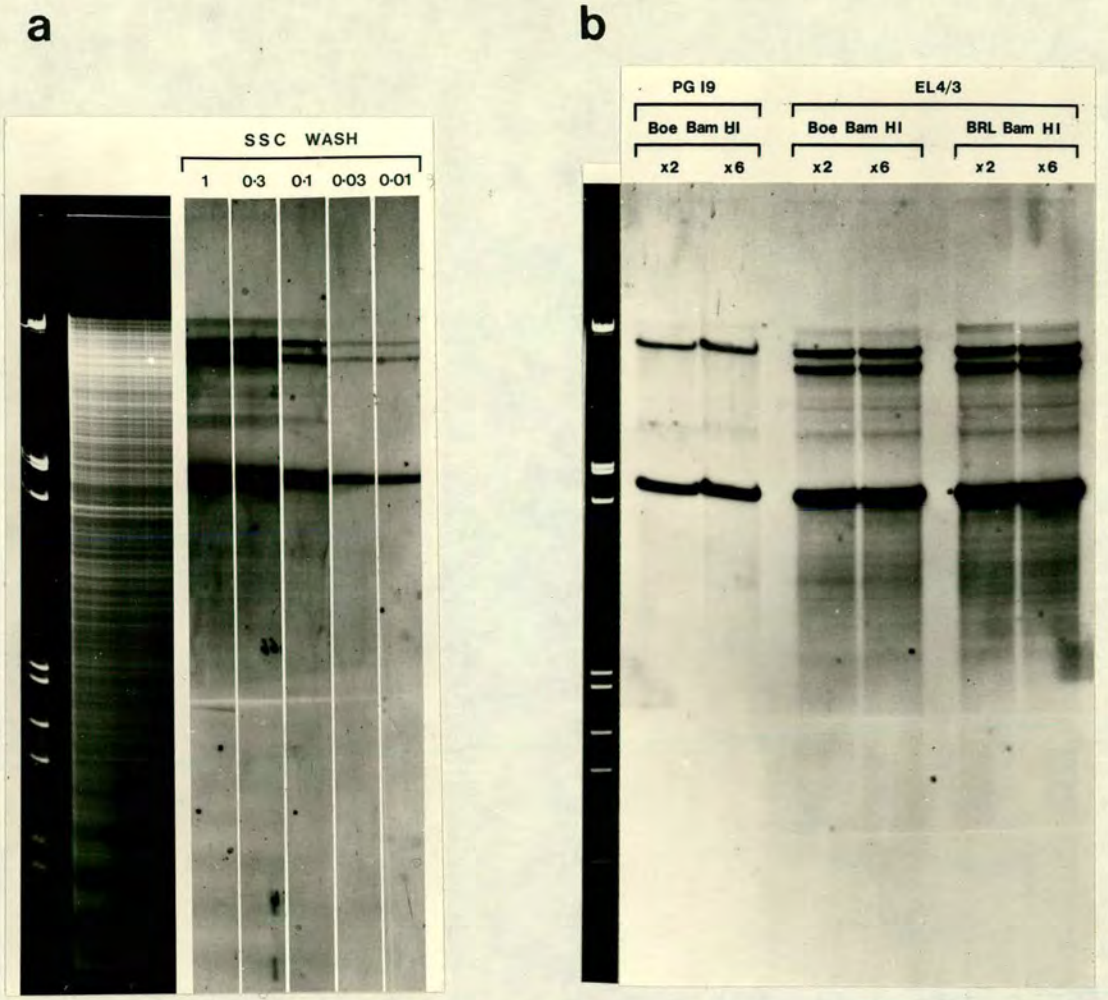


Fig. 3.15. Analysis of the multiple feint bands in MTX-resistant cell DNA which hybridize with pDHFR 21.

a. EL4/3:$MTX_R^{2.2x10^{-3}M}$ cell DNA was digested with Bam HI and electrophoresed in a 1% agarose gel. After transfer to nitrocellulose and hybridization with the Pst I-excised DHFR cDNA insert from pDHFR 21 labelled with ^{125}I by nick-translation, strips of filter were washed at 65°C in the concentration of SSC shown at the top of each track. On the left-hand side of the figure are shown part of the ethidium bromide-stained gel and the marker DNA.

b. PG19T3:$MTX_R^{10^{-4}M}$ S1 DNA (PG19) or EL4/3:$MTX_R^{2.2x10^{-3}M}$ DNA (EL4/3) were digested with Bam HI from Boehringer-Mannheim (Boe) or Bethesda Research Laboratories (BRL) using either a 2-fold excess of enzyme (x2) or a 6-fold excess of enzyme (x6). Fragments were electrophoresed in a 1% agarose gel and transfered to nitrocellulose. The filter was hybridized with the ^{125}I-labelled Pst I-excised DHFR cDNA insert from pDHFR 21 and autoradiographed. Note that essentially the same minor bands can be seen in each EL4/3 track.

low degree of homology but are present in many copies per haploid genome. To distinguish between these possibilities, hybrids formed between genomic DNA bound to the nitrocellulose and the pDHFR 21 insert in solution were washed under different conditions of stringency. If the feint bands represent homologous hybrids, they should show the same proportional decrease in intensity as the authentic DHFR bands when stringency is increased. If they represent heterologous hybrids, they should show a larger proportional decrease in intensity when stringency is increased. So far as can be determined from fig. 3.15.a., both the authentic DHFR bands and the extra feint bands show the same proportional changes in intensity. To this conclusion must be added the proviso that the blackening of the X-ray film may not be directly proportional to radioactivity over such a large range of intensities. However, the most important observation is that the intensity of these feint bands is not enormously greater in strips of filter which have been washed in 1 x SSC than in strips given the standard wash of 0.1 x SSC. If the feint bands represented multiple copies of a sequence with a small degree of homology with DHFR they should be proportionally more intense after a lower stringency wash. Thus the feint bands appear to be genuine DHFR sequences.

Fig. 3.15.b. shows an investigation of the possibility that the feint bands are an artefact generated by incomplete specificity of the restriction enzyme preparation. Such decreased specificity could arise in two ways. A Bam

HI* activity could be present, analagous to the Eco RI* activity observed under suitable salt conditions, where the specificity of Eco RI cleavage decreases from GAATTC to NAATTN. Alternatively it could be that Bacillus amyloliquefaciens H contains other, previously undetected, restriction enzymes: a hypothetical Bam HII. A small amount of either contaminating activity could produce a series of minor bands such as those observed. If such contaminating activity is responsible for the feint bands then it should be manifested on overdigestion. Increasing digestion beyond that necessary to cleave at all Bam HI sites should not alter the Bam HI pattern, but it should increase the amount of any contaminating cleavage. Fig. 3.15b. shows that overdigestion does not lead to an increase in the intensity of the feint bands. Therefore they are not artefacts generated by additional restriction enzyme cleavage within the DHFR bands.

It therefore appears that the amplified DHFR genes in MTX-resistant cell DNA are present in the restriction fragments characteristic of wild-type cells, but also in an additional heterogeneous set of fragments. The true origin of these heterogeneous feint bands became clear when endogenous DNase activity in the MTX-resistant cell nuclei was examined. Isolated nuclei from PG19T3: MTX_R 10^{-4}M S1 cells were incubated at 37°C in 10mM Tris-HCl pH 7.4, 10mM NaCl, 3mM $MgCl_2$ for varying times before DNA was prepared and digested with Bam HI. The patterns of fragments in total DNA and those containing DHFR genes in these

samples are shown in fig.3.16. It can be seen from fig. 3.16a. that the state of overall DNA degradation in these nuclei is small; hardly any difference can be detected between the size distribution of total DNA visualised by staining with ethidium bromide in the '0 min' and '30 min' samples. In contrast fig.3.16b. shows that considerable degradation of the DHFR gene has occurred in this time. Of the three bands represented in undegraded DNA, the largest is undetectable in DNA after 30 minutes incubation at 37°C and the smaller two have decreased considerably in intensity. In addition, hybridization is seen to several discrete bands of new sizes and there is an increase in overall background in the track. The most prominent new bands form two clusters at about 8.5Kb and 7Kb; each cluster can be seen to be made up of at least three bands of similar size. Discrete bands are not seen when no restriction enzyme is used; therefore each of the fragments in these new bands must be bounded at one end by a Bam HI site and at the other by a DNase site.

Overexposure of the autoradiograph (fig.3.16c.) shows that the new bands seen clearly in the 30 minute lane in fig.3.16b. can just be detected in the 0 minute lane in fig.3.16c. It therefore appears that, even in a DNA preparation fron nuclei which have not been incubated under conditions in which endogenous nucleases might be activated, a small amount of degradation has occurred, which results in the appearance of additional bands upon subsequent digestion with restriction endonucleases.

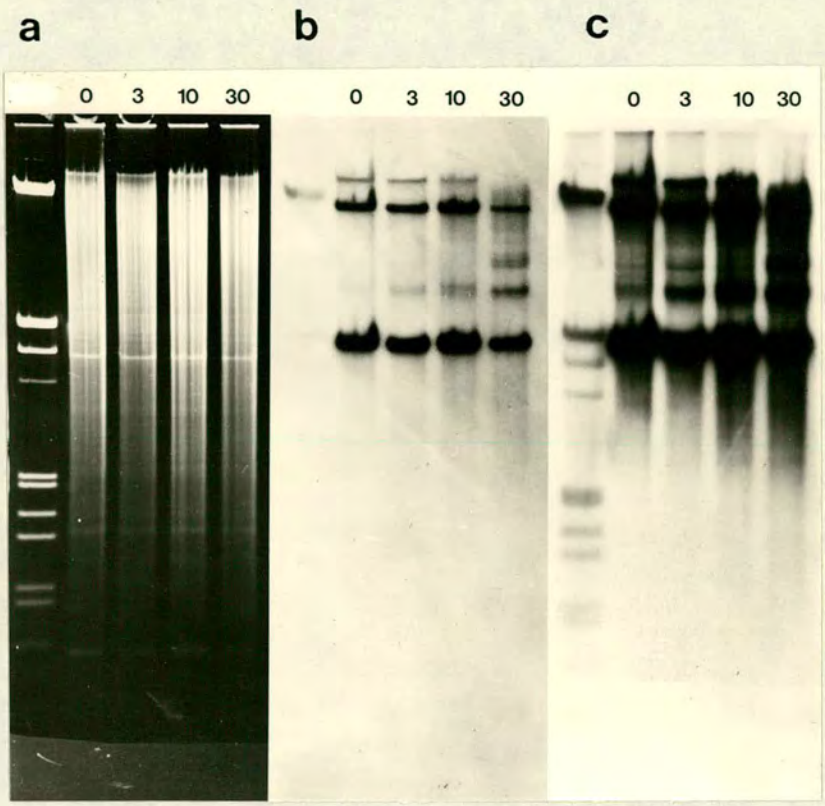


Fig. 3.16. Origin of the heterogeneous feint DHFR bands in MTX-resistant cell DNA.

PG19T3:MTX_R 10^{-4}M S1 cell nuclei were incubated at 37°C in 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$ for the times shown (in minutes) at the top of each track. DNA was prepared, digested with Bam HI, and electrophoresed in a 0.75% agarose gel.

a. The gel was stained with ethidium bromide, illuminated with u/v lamps, and the fluorescence was photographed.

b. DNA was transfered to nitrocellulose, the filter was hybridized with ^{32}P-labelled pDHFR 21,and autoradiographed for about 14 hours.

c. The same filter was autoradiographed for about 150 hours.

A comparison of fig. 3.16. with fig.3.12d. shows that the bands generated by nuclease cleavage at such sites in chromatin accounts for some of the aberant new bands which are seen in resistant cell DNA. Other bands, however, cannot have arisen by such cleavage. It can be seen in fig.3.16b. that DNase degradation of the highest molecular weight Bam HI band (measured as 58Kb on other gels) never produces another high molecular weight band (about 33Kb) as seen in the EL4/3:$MTX_R^{2.2x10^{-3}M}$ DNA track in fig.3.12d., either when about half the 58Kb band is degraded (10 minutes incubation) or when essentially all of it is degraded (30 minutes incubation). Similarly, DNase degradation for any length of time does not generate a prominent band at about 13Kb; a band of this size and almost equal in intensity to the 17Kb band common to all cell lines can be seen in the EL4/3:$MTX_R^{2.2x10^{-3}M}$ DNA track in fig.3.12d.

Thus, in addition to the new bands generated by endogenous DNase cleavage, there appears to be another class of extra band, for which the 33Kb and 13Kb Bam HI bands in EL4/3:$MTX_R^{2.2x10^{-3}M}$ DNA are the prototypes. This class is distinguished from the DNase generated bands by some or all of the following characteristics.

1. Bands do not increase in intensity when nuclei are incubated at 37°C before DNA is isolated.
2. Bands are unique to a cell line; whereas the DNase generated bands can be seen in suitable exposures of all cell lines.

3. Bands are, when some restriction enzymes are used, larger than other DHFR hybridizing bands; DNase degradation must necessarily produce smaller bands.

3.10. EXTENSIVE DNA REARRANGEMENT HAS OCCURRED NEAR THE ENDS OF SOME OF THE DHFR GENES IN THREE OUT OF FOUR MTX-RESISTANT EL4 CELL LINES

Category 2 bands can be seen in EL4/3:$MTX_R^{2.2x10^{-3}M}$ DNA digested with Bam HI, with Pst I, and possibly with Eco RI (fig 3.12d-f). In the last case the band is feint and is situated close to an intense band, but is reproducibly present in replicate digests of the same or independent DNA preparations from this cell line. In addition, new bands can be seen in Hind III and Acc I digests (fig.3.17.) A single base change is sufficient to create or destroy a restriction enzyme site, but the finding of new restriction fragments with several enzymes would, if they were all generated by single base changes, imply a very high frequency of point mutation in this cell line. A more likely explanation is that DNA rearrangement has led to the replacement of a stretch of wild-type DNA, containing the restriction sites observed in EL4/WILD digests and most MTX-resistant cell lines, with a new stretch of DNA containing the new restriction sites seen only in EL4/3:$MTX_R^{2.2x10^{-3}M}$ DNA. Such a rearrangement must affect only a proportion of the DHFR genes in the cell, or only a proportion of the cells in the population, since the wild-type bands are always seen in addition to

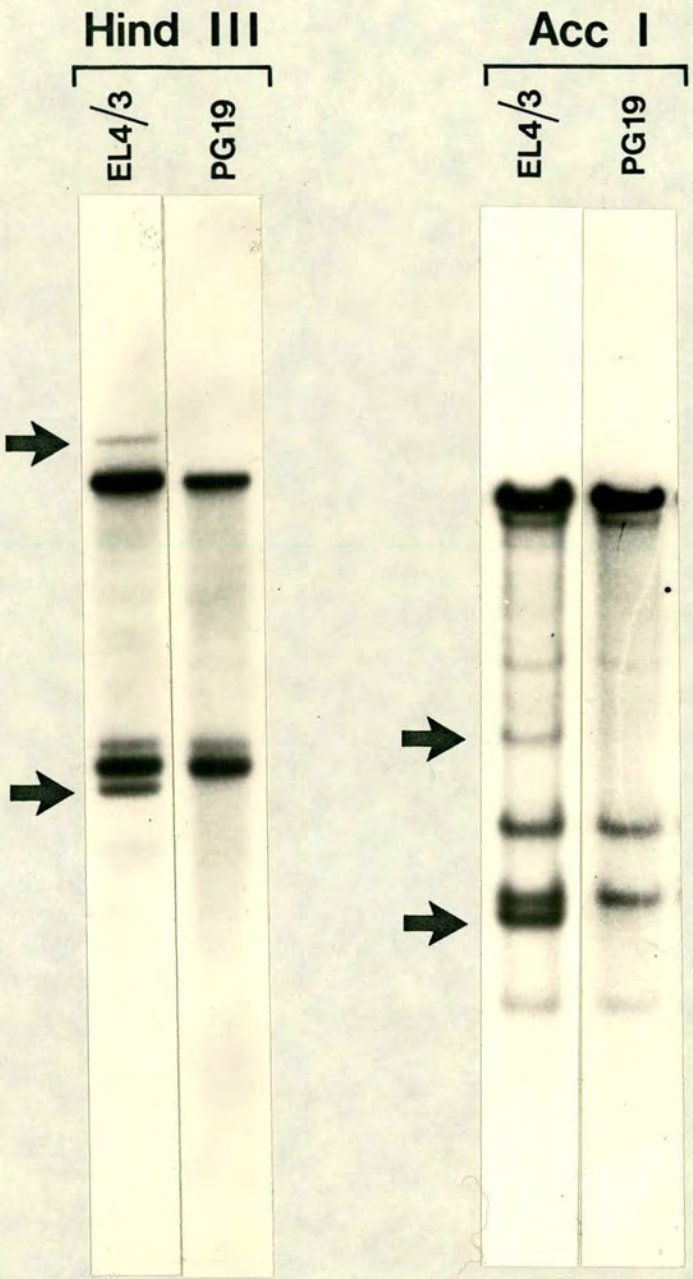


Fig. 3.17. Analysis of the DHFR band pattern in EL4/3 cells using Hind III and Acc I. DNA from EL4/3:$MTX_R^{2.2\times10^{-3}M}$ cells or from PG19T3:$MTX_R^{10^{-4}M}$S1 cells (representing the wild-type pattern) was digested with the enzyme shown at the top of the track, electrophoresed in a 0.75% agarose gel and transfered to nitrocellulose. The filter was hybridized with pDHFR 21 and autoradiographed. Bands seen in EL4/3 DNA but not in PG19 or any other sample are arrowed.

any new bands.

Fig.3.18. shows the patterns of DHFR-containing fragments in EL4/3:$MTX_R^{2.2x10^{-3}M}$ DNA digested with Hae III and Taq I, and compared with the wild-type pattern which is represented here by PG19T3:$MTX_R^{10^{-4}M}$ DNA. The patterns seen seen in the two cell lines are identical. This shows that the DNA rearrangement which has given rise to the new restriction fragments seen in figs. 3.12 and 3.17. can be separated from the sequences detected by hybridization with pDHFR 21, which detects only the exon sequences of the DHFR gene; therefore the rearrangement has occurred outside the exon regions and is either in the introns or in the sequences which flank the DHFR gene.

In order to investigate whether DNA rearrangement is a common feature of MTX-resistant EL4 cell lines, or is peculiar to EL4/3 cells, DNA from three independently selected MTX-resistant EL4 lines has been examined and the results shown in figs.3.19. and 3.20. In fig.3.19. it can be seen that one novel band is visible in each of the Eco RI digests of DNA from EL4/8:$MTX_R^{1.1x10^{-3}M}$ cells and EL4/11:$MTX_R^{1.1x10^{-3}M}$ cells. These novel bands are analogous to the EL4/3:$MTX_R^{2.2x10^{-3}M}$ cell extra bands in that new bands are detected with more than one enzyme (fig.3.20.), suggesting that DNA rearrangement, rather than point mutation, is the cause, and in that only a proportion of the DHFR genes in each cell line have been affected. The DNA rearrangement is different in each of the three cell lines. The fourth of these MTX-resistant cell lines,

EL4/12:$MTX_R^{1.1x10^{-3}M}$, shows only the wild-type DHFR gene pattern with all enzymes tested. In EL4/8:$MTX_R^{1.1x10^{-3}M}$ DNA double-digested with both Eco RI and Bam HI, the extra band is different in size to those seen in either of the Eco RI or Bam HI single digests of this DNA, thus showing that, at least in this cell line, the same region of rearranged DNA is being detected by both enzymes.

The rearranged DNA has been localised to subregions of the DHFR gene by using fragments of pDHFR 11 as probes (fig.3.20.). A Pst I-Hae III fragment from the 5' end of the DHFR gene detects the 33Kb Bam HI extra band in EL4/3:$MTX_R^{2.2x10^{-3}M}$DNA. A Bgl II fragment from the 3' end of the gene detects extra fragments in Bam HI digests of EL4/3:$MTX_R^{2.2x10^{-3}M}$ DNA, EL4/8:$MTX_R^{1.1x10^{-3}M}$ DNA and EL4/11:$MTX_R^{1.1x10^{-3}M}$ DNA, and extra fragments in Eco RI digests of EL4/8:$MTX_R^{1.1x10^{-3}M}$DNA and EL4/11:$MTX_R^{1.1x10^{-3}M}$DNA. These two subprobes together account for all the extra bands seen in fig.3.20. when complete pDHFR 21 was used as probe; no additional bands were seen when Hae III fragments from the middle of pDHFR 11 were used (results not shown). The feint Eco RI band in the EL4/3:$MTX_R^{2.2x10^{-3}M}$DNA was not detected in this experiment. Thus DNA rearrangement in these three EL4 cell lines is located near the ends of the DHFR gene. From the results shown in fig.3.20. it is not possible to determine whether the rearrangement has occurred in the introns nearest to the 5' or 3' ends, or in the sequences which flank the gene.

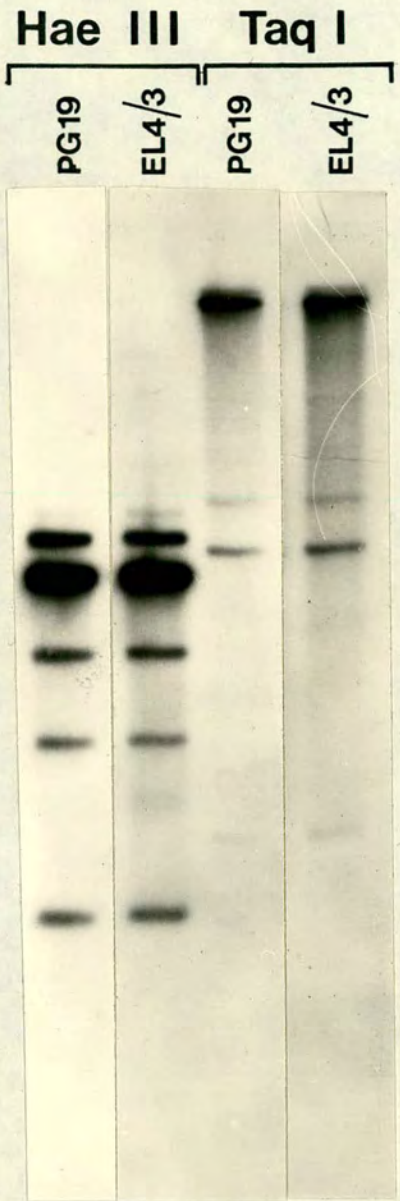


Fig. 3.18. Analysis of the DHFR band pattern in EL4/3 cells using Hae III and Taq I. A procedure identical to that described in the legend to fig. 3.17. was followed, except that a 1% agarose gel was used. Note that the band patterns in the different DNA preparations are identical.

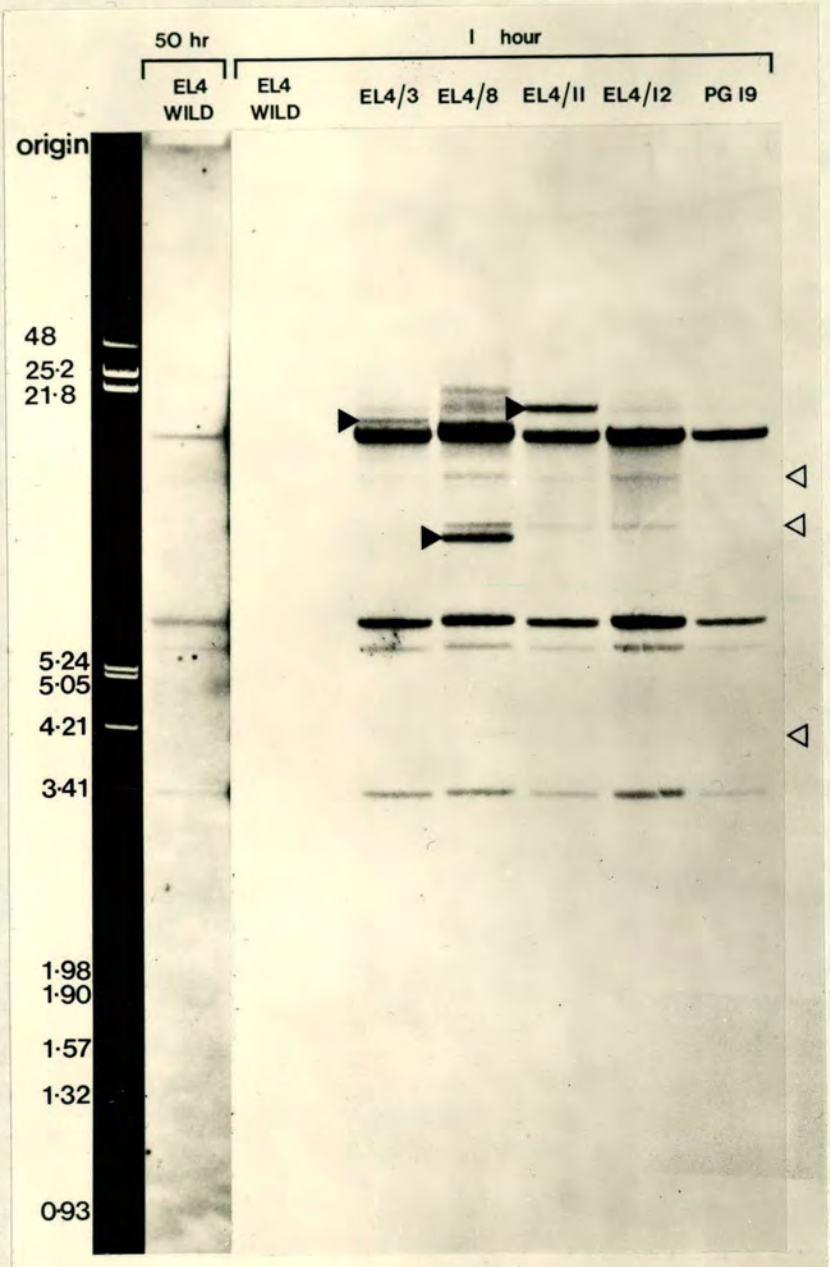


Fig. 3.19. Analysis of the DHFR band patterns in EL4 cell DNA.
DNA samples were digested with Eco RI and fractionated in a 0.75% agarose gel. DNA was transfered to nitrocellulose, the filter was hybridized with pDHFR 11 in the presence of 10% dextran sulphate, and autoradiographed for 1hr or 50hrs as indicated. Open arrows (▷) mark the positions of bands thought to be generated by cleavage at hypersensitive sites in chromatin by an endogenous DNase and present in all samples. Closed arrows (▶) indicate the positions of bands thought to result from DNA rearrangement and unique to a sample.

Fig. 3.20. Mapping of the DHFR restriction fragments in EL4 cells.

A. Partial restriction map of pDHFR 11 drawn using data in ref [123]. The thick line represents part of the pBR322 plasmid vector, the open bar represents the coding portion of the DHFR cDNA and the thin line represents the 5' and 3' non-coding portions of the cDNA.

B. Location of the Pst I - Hae III fragment used to probe the 5' end of the gene and the Bgl II fragment used to probe the 3' end of the gene.

C. DNA samples were digested with restriction enzymes as indicated, electrophoresed in a 1% agarose gel and transfered to nitrocellulose. The filter was hybridized with the ^{32}P-labelled probe shown in part B and autoradiographed. Open arrows (▷) indicate bands thought to be generated by cleavage at hypersensitive sites in chromatin by an endogenous DNase. Closed arrows (▶) indicate bands thought to result from DNA rearrangement. The DNA samples are identified as follows:

PG19 = PG19T3:$MTX_R^{10^{-4}M}$

3 = EL4/3:$MTX_R^{2.2\times10^{-3}M}$

8 = EL4/8:$MTX_R^{1.1\times10^{-3}M}$

11 = EL4/11:$MTX_R^{1.1\times10^{-3}M}$

12 = EL4/12:$MTX_R^{1.1\times10^{-3}M}$

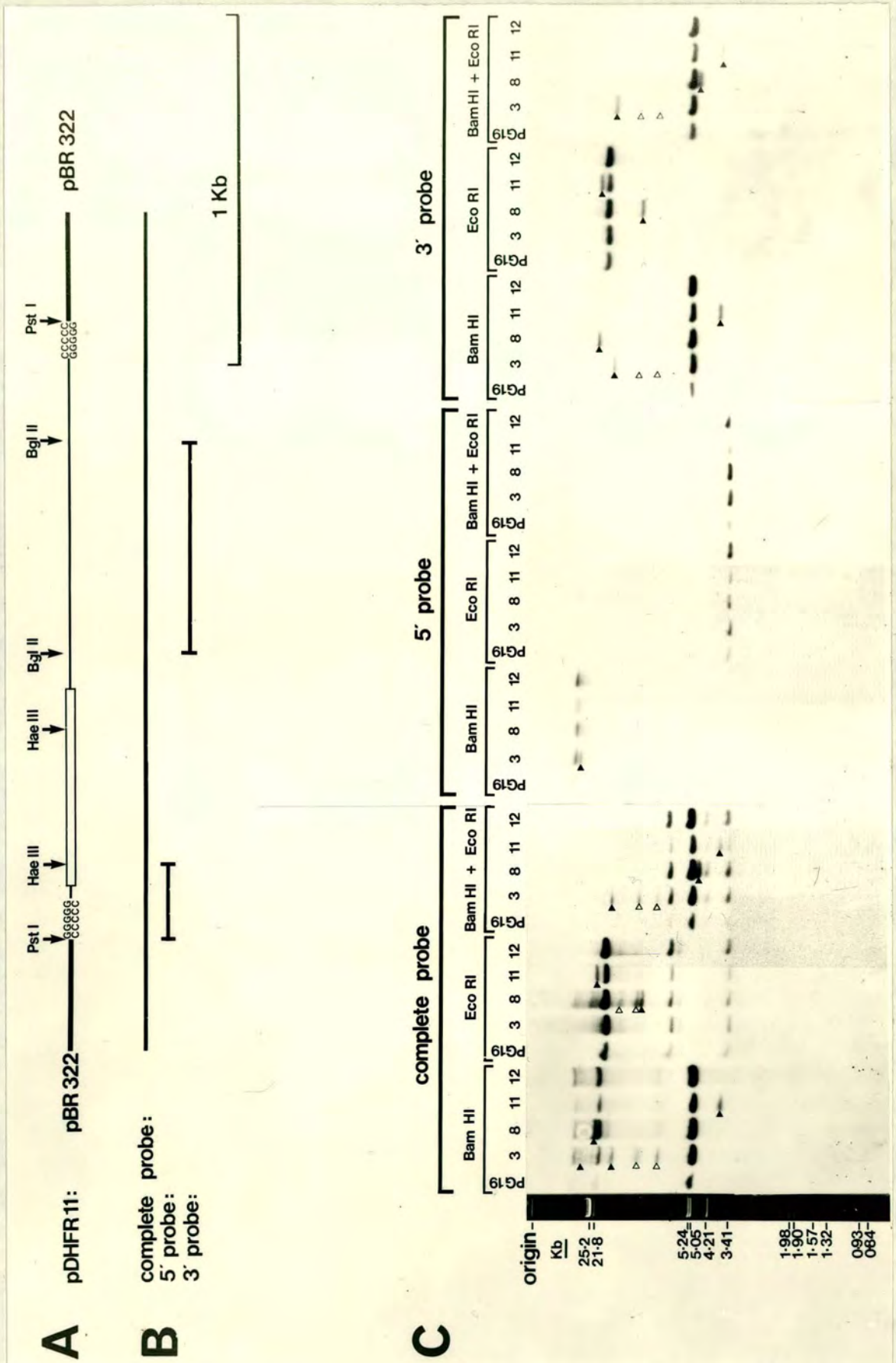


Fig. 3.20. Mapping of the DHFR restriction fragments in EL4 cells.

See opposite page for legend.

CHAPTER 4

DISCUSSION PART I

4.1. SCOPE OF THIS DISCUSSION

In chapter 3 it was found that the MTX-resistant cell lines characterized showed considerable overproduction of DHFR enzyme, and that the basis for this overproduction was the increase in DHFR RNA associated with DHFR gene amplification. However, the numerology was not straightforward; that is, a cell line which was 1000-fold resistant to MTX did not show 1000-fold overproduction of DHFR enzyme and 1000-fold amplification of the DHFR gene. A discussion of possible reasons for the more complex relationships which were observed forms the first half of this chapter. The second half is devoted to a discussion of the DNA rearrangement which has accompanied DHFR gene amplification in some EL4 cell lines. Specifically omitted from this chapter, however, is a general discussion of the amplification process; this can be found in chapter 6.

4.2. MTX RESISTANCE LEVEL AND DHFR OVERPRODUCTION

The results shown in fig.3.2. indicate that there is not a simple linear relationship between MTX resistance level and DHFR overproduction. The factors which might be expected to influence the relationship fall into several categories. There are, in addition to DHFR overproduction, other mechanisms by which cells become resistant to MTX. These were described in section 1.4. and their possible occurrence in the cell lines characterized in chapter 3 will be discussed at the end of this section. However, even if alterations in MTX transport and changes in

DHFR structure have not contributed to MTX resistance in these cells, there are additional reasons for expecting a non-linear relationship between MTX resistance level and DHFR overproduction.

It is important to distinguish between comparisons of absolute values for different MTX resistant cell lines and comparisons of resistant cells relative to their sensitive counterparts; that is, between the absolute DHFR specific activity and MTX concentration, and the relative DHFR overproduction and increase in MTX resistance. Absolute DHFR specific activities have been listed in table 3.1. It can be seen that there is considerable variation between the specific activities measured in different wild-type cell lines. This variation could reflect either different numbers of DHFR molecules per cell, or different amounts of total protein per cell, or both. The cell lines have been derived from different tissues in different mouse strains. It is not known whether one DHFR gene always produces the same number of DHFR molecules, or whether expression depends on the environment. In the former case, variation in expression of other genes, leading to different amounts of protein per cell, would be the explanation for the variation in specific activity; in the latter case, variation in DHFR gene expression would also contribute. "Induction" of human DHFR has been reported (81,82), but it is not known whether a similar phenomenon occurs in the mouse.

DHFR specific activities for MTX-resistant cell lines are listed in table 3.1. Because instability in some cell lines makes extrapolation of the data back to day 0 necessary, there may be considerable errors in these estimates. The increase in DHFR specific activity (DHFR overproduction) has been calculated by dividing the specific activity estimated in resistant cell extracts by the specific activity measured in wild-type cell extracts. Therefore it does not necessarily reflect the increase in the cells' capacity to produce DHFR, since it does not allow for any increase which might occur on "induction" of wild-type cells. In order to obtain a true measure of the increase in the cells' capacity to produce DHFR, the DHFR activity of resistant cells grown in that concentration of MTX which just does not inhibit their growth. It is impossible to obtain these measurements because DHFR activity must be measured in cells which have been grown in the absence of MTX; nevertheless, it is a limitation on the interpretation of DHFR overproduction estimates.

should be compared with the DHFR activity of wild-type cells grown in that concentration of MTX which just does not inhibit their growth.

The absolute MTX concentrations in which the MTX-resistant cell lines are routinely grown are shown in table 3.1. The degree of MTX resistance is assessed by comparing the concentrations of MTX which halve the cloning efficiencies (relative to those in non-selective medium) of resistant and sensitive cells. It is this measurement of resistance which should be compared with the increase in the cells' DHFR activity. Data on the plating efficiency of sensitive and resistant PG19 cells in medium containing MTX is

available (94) but measurements for the other cell lines have not been made. In the case of the PG19 cells, an approximately 30,000-fold increase in MTX resistance was accompanied by an approximately 1,000-fold increase in DHFR specific activity. When the DHFR specific activity measured at successive stages in the selection was compared with the MTX concentration in the selective medium, a relationship of the form

$$\text{DHFR specific activity} \propto [\text{MTX}]^{0.65}$$

was found. The fact that this relationship was observed at all stages in selection suggested that no change in the mechanism of resistance had occurred during selection. Several explanations for the fact that the exponent is less that one are possible: more than one MTX molecule may be necessary to inhibit DHFR *in vivo* (cf *in vitro*), or the MTX concentration inside the cell may be less than the concentration outside because of decreased influx or increased efflux, or the concentration of DHF may have increased, thus competing more effectively with the MTX.

For the other cell lines, where the increase in MTX resistance over wild-type is unknown, the most meaningful comparison which can be made is that of the absolute DHFR specific activity with the absolute MTX concentration (fig.3.2a.). Since these are not related in a simple fashion, factors other than DHFR activity, such as alterations in MTX transport or DHFR structure (see chapter 1), must be important in determining resistance. No DHFR protein with altered structure and MTX-binding properties has been

reported from mouse cells (section1.4.). While it is possible that L1210:$MTX_R^{10^{-4}M}$ cell DHFR characterized here (sections 3.3. and 3.4.) may represent the first example of such an alteration, this could not be confirmed by comparing MTX inhibition of DHFR <u>in vitro</u> in extracts of sensitive and resistant L1210 cells since no sensitive L1210 cells were available for study. No differences in the DHFR characteristics studied were found in the other MTX-resistant cell lines.

If the DHFR enzyme properties are the same in sensitive and resistant cells, it is likely that the differences in MTX transport between different MTX-resistant cell lines contribute to the scatter of points seen in fig.3.2a. In one case it has been shown by Courtenay and Robbins (75) that the L5178Y:$MTX_R^{1.1x10^{-3}M}$ cell line, from which the L5178Y: $MTX_R^{4.4x10^{-3}M}$ cell line was derived, has reduced MTX uptake. In other cases this has not been demonstrated. However, in particular, the low DHFR specific activity and high MTX resistance level of LB1 trans: $MTX_R^{4.4\times10^{-3}M}$ cells suggests that these cells have reduced transport.

It is instructive to compare the results reported here with those in the literature. Some examples of the relationship between MTX resistance level and DHFR specific activity have been given in chapter 1. In addition, the results of Biedler, Spengler and co-workers on anti-folate resistant Chinese hamster cells (68-71) have been collected and displayed in a form similar to fig 3.2a. in a more recent publication. (1 They have included data on 22 resistant cell lines derived

from a single starting line, 20 of which were selected independently. There is such a wide scatter of points in their fig 1 that no clear relationship between MTX resistance level and DHFR activity can be seen, although Biedler et al discern three groups of points, of which one has an increase in MTX resistance relative to DHFR overproduction of 1.6; a second, a relative increase of about 46, and a third a relative increase of about 520. The molecular basis for these classes is unknown, but the wide scatter of points suggests that the kind of variation illustrated in fig.3.2a. will generally be found when different MTX-resistant cell lines are compared.

4.3. DHFR ENZYME OVERPRODUCTION AND DHFR GENE AMPLIFICATION

Fig.3.11.6 shows a plot of DHFR enzyme overproduction against estimated DHFR gene number per 3×10^6 Kb of DNA. For some cell lines, two estimates of gene number have been included; that obtained by spot hybridization, and that obtained by blot quantitation. It can be seen that in each case the two estimates for the same cell line are different, and that blot quantitation always gives the lower figure.

The first question to be considered in this section is therefore why the two estimates should differ. The answer is likely to be that the two methods measure different things: spot hybridization measures the total amount of DNA which cross-hybridizes with the probe, whereas

blot quantitation measures only that proportion of the total hybridization which is contributed by specific restriction fragments. Part of the difference between the estimates can be accounted for by the imprecision inherent in the techniques, but part is likely to be real. Cross-hybridization of the probe to non-DHFR sequences could contribute to spot hybridization estimates: for example, mouse satellite DNA cross-reacts with pDHFR 21 to a very small extent (Noel Ellis, personal communication). However, the most important factor is probably cleavage of the DHFR gene by endogenous DNase (section3.9.). This would lead to loss of hybridization from the specific bands and to an increase in the background in the remainder of the gel track. This would not be included in blot hybridization, but would contribute to estimates based on spot hybridization. What is the reason for this DNase cleavage?

It is known that MTX resistance in some cell lines is unstable (75, 129), such that, after growth in the absence of MTX, cells can no longer survive in the original MTX concentration. By this criterion, some of the cell lines characterized here are highly unstable (see chapter 5). In addition, during growth under selective conditions, cells which have lost the ability to exclude vital stains are readily detected in these lines. It would seem that, even under constant selection, cells which have lost the ability to grow in MTX are continually being generated. DNA degradation will eventually occur in such cells.

Living and dead cells are not readily separated from each other in the suspensions of EL4 cells, so that when DNA is prepared from the population it is found to consist of an intact fraction derived from living cells and a degraded fraction from dead and dying cells.

Some support for this hypothesis comes from a comparison of the different MTX-resistant EL4 cell lines. Resistance in EL4/11:$MTX_R^{1.1x10^{-3}M}$ cells is stable as judged by DHFR enzyme activity measurements (C.J.Bostock, personal communication) and estimation of DHFR gene number (section 5.5.) of cells grown under non-selective conditions, while resistance in the other three EL4 lines is unstable as judged by the same criteria (C.J.Bostock and section 5.5.). The two estimates of gene number for the EL4/11:$MTX_R^{1.1x10^{-3}M}$ cells differ by only a few per cent, a difference which can be accounted for by technical limitations on accuracy. In contrast, the estimates for the other three lines differ by two- to three-fold, which is significantly more than the error in these measurements.

Which of the two measurements more accurately reflects the number of DHFR genes? For a cell line in which resistance is stable, it does not matter, because they are essentially the same. For an unstable cell line, blot quantitation estimates the number of intact and therefore potentially functional, DHFR genes. However, in blot quantitation experiments, only the total amount of DNA is known, and not that proportion of the

DNA which gives rise to intact gene bands. Therefore blot quantitation will tend to underestimate the number of DHFR genes, while spot hybridization will give a better estimate of the total number. The two estimates will be useful for different purposes.

Using such estimates, it is now possible to turn to a discussion of the relationship between DHFR gene number and DHFR enzyme overproduction, as shown in fig.3.11.6 It can be seen that there is no simple linear relationship between the two measurements. In general, the cell lines in which MTX resistance is unstable (EL4/3, 8, 12) have the highest numbers of DHFR genes. A possible reason for this is that in these cells many of the DHFR genes may not be located in the nucleus and may therefore be transcribed, or the RNA processed or exported, at a lower rate than the nuclear genes, although there is no direct evidence for such differential expression. The evidence that some DHFR genes are extra-nuclear is indirect and depends on interpretation of the following cytological data. In the unstable cell lines, many micronuclei can readily be observed in interphase cells (C.J.Bostock, personal communication). These are thought to arise from chromatin fragments which lack functioning centromeres and therefore do not segregate normally and equally at mitosis. When the nuclear membrane reforms at the end of telophase, some of these fragments can be left in the cytoplasm and give rise to the

micronuclei during interphase. Evidence that some DHFR genes are associated with such chromosomes is presented in section 5.5. However, nothing is known about gene expression in such micronuclei (and see the discussion on DHFR gene activity below).

A second factor which might account for the very high number of DHFR genes found in the MTX-resistant EL4 cell lines is that the DNA rearrangement observed in three of the cell lines near the DHFR gene has led to the formation of a non-functional DHFR gene, or one with a lower rate of transcription. These rearranged genes will contribute to estimates of gene number, but may not contribute to DHFR enzyme activity. However, the high number of DHFR genes in EL4/12:$MTX_R^{1.1x10^{-3}M}$ cells, which do not have detectably rearranged DNA near the DHFR gene but are unstably resistant, and the lower number of DHFR genes in the EL4/11:$MTX_R^{1.1x10^{-3}M}$ cells which do have rearranged DNA but are stably resistant, suggests that stability is the more important factor in producing MTX resistance. The high number of genes asociated with unstably resistant cells may arise in the following way. If DHFR genes segregate unequally, then one daughter cell will tend to have more genes than the number required for survival in a given MTX concentration, and the other daughter cell will tend to have less. Any cell with too few genes will be killed, and consequently there will tend to be an excess of genes in the population.

Even amongst the non-EL4 cell lines, however, the relationship between DHFR gene number and DHFR enzyme overproduction is not simple. One complication arises because estimates of the DHFR gene number have been calculated using a nominal haploid genome size of 3×10^6 Kb. The real amount of DNA per cell may not be 6×10^6 Kb, and may differ in different cell lines because of aneuploidy and amplification of a different sized unit. Thus, the estimated number of DHFR genes may not be the true number. Also, in these cell lines additional factors such as the rate of transcription, or the mRNA stability or rate of translation, or enzyme stability, may be different. While some evidence suggests that sequences in the amplified unit differ from cell line to cell line (section 5.2.) and therefore could produce different rates of transcription, nothing is known about the other factors.

DHFR gene numbers have only been estimated for a few other cell lines, and published data are summarised in table 4.1. It can be seen that, while in some of these cell lines there is a one-to-one correlation between DHFR overproduction and increase in DHFR gene number, in others, as in the cell lines described in this thesis, there is not such a simple relationship.

Strictly, there is no evidence from any cell line described here or elsewhere that more than one DHFR gene per cell is active. DHFR overproduction could

Species	Cell line	Number of DHFR genes	DHFR enzyme overproduction	Reference
Mouse	S-180	200	200	87
		180	250	236
	L1210	45	35	87
	L5178Y	"several 100"	300	140
		400	350-400	236
	3T6-R1	35	33	237
	3T6-R2	100	100	237
	3T6	75	50-100	236
Hamster	BHK	200	140	236
	CHO	160	150	236
	DC-3F/MQ19	120	150	238
	DC-35/A3	53	170	238
	DC-35/MQ20	20	49	238
	A3-P-U	7	18	238

Table 4.1. Published reports of DHFR gene number and corresponding DHFR enzyme overproduction in MTX-resistant cell lines.

be due to an increased rate of expression of a single gene in the resistant cell, rather than expression of many genes at the wild-type rate. The amplified copies of the DHFR gene which are associated with overproduction could mediate the increase in DHFR synthesis by increasing the rate of expression of the one gene, for example, by binding a (hypothetical) DHFR gene repressor molecule.

Two measureable properties of DNA sequences, which have been widely studied in the hope of elucidating gene expression, are methylation and DNase sensitivity. Such work on gene expression in mammals has concentrated exclu--sively on the few developmentally regulated genes which code for major cell proteins and for which nucleuc acid probes have been available. The relevance of results obtained in such systems to the expression of a "housekeeping" gene such as DHFR is questionable. Despite this reservation it can be asked whether the results described in sections, 3.8. and 3.9. throw any light upon DHFR gene activity. In general, a correlation has been found between demethylation and transcription or transcribability. This has been established for β-type globin genes in rabbit (130), chicken (131) and man (132), and for ovalbumin, conal--bumin and ovmucoid genes in the chicken (133,134,). In all cases only a proportion of the 5mC residues in the region of the gene become demethylated when the gene is activated, and often demethylation at an affected site is only partial. Thus, although the DHFR genes in both MTX-resistant and wild-type cells are extensively methylated

it is possible that this observation does not conflict with the established dogma that active genes are demethylated. Firstly, only some of the CpG sequences have been examined - those which occur in the restriction sites of the enzymes used - and these may not be the ones important for expression; secondly, some demethylated sites were found, and it is possible that this small observed degree of demethylation, or that which may exist in the sites not examined, may be sufficient for transcription.

A second kind of correlation has been found in several systems: that between the DNase I sensitivity of a gene and its transcribability (135,134). DNase sensitivity in this sense has not been examined in the DHFR gene. The phenomenon discovered here, hypersensitivity to cleavage by an endogenous DNase, is analagous rather to DNase I hypersensitivity. DNase hypersensitivity was first found near the heat-shock genes of Drosophil a melanogaster (136, 137, 138) and has since been observed near chicken conalbumin (134) and chicken globin (139) genes. While the early evidence from the heat-shock genes of the fly seemed to show a correlation between the presence of the hypersensitive sites and inactivity, and between their disappearance and activation (137), the site which was found about 1 Kb downstream from the 3' end of the chicken conalbumin gene was present whether the gene was active or not (134), and different hypersensitive sites were found near chicken globin genes in different states of activity in embryos and adults (139). Thus the

relationship of such sites to gene expression is at present unclear. The molecular basis for these sites is also unclear. They could be due to peculiarities of the DNA structure, such as a particular sequence or a single-stranded nick, or they could be due to the presence of specific protein species or configurations in the chromatin. These possibilities can be distinguished experimentally, but no results from such experiments have yet been published.

In conclusion, it can be said that while the DHFR gene appears to have some properties of inactive genes - extensive methylation and DNase hypersensitivity - too little is known at present about gene expression to decide whether or not most of the amplified DHFR genes are expressed.

4.4. DNA REARRANGEMENT NEAR THE DHFR GENE

It was shown in section 3.10. that DNA rearrangement had taken place in three out of four MTX-resistant EL4 cell lines, and that this rearrangement was located near the ends of the gene: either in the 5' and 3'-most introns or in the sequences which flank the gene. This result was striking because none of the other MTX-resistant cell lines described in section 3 or by others (123) showed evidence of DHFR gene rearrangement, although there was some ambiguity over the size and position of Bam HI fragments in MTX-resistant S-180 cells (123, see note added in proof). Thus DNA rearrangement in the vicinity of the

DHFR gene does not appear to be a general feature of gene amplification.

It was not known at which stage in the selection of the EL4 cells the rearrangement took place. Cells resistant to intermediate levels of MTX were not frozen down, and so this question could not be investigated experimentally by examining DNA from such cells. However, analysis of DNA from the final stage of resistance shows that the amount of hybridization to the rearranged fragments is relatively high: in EL4/8:MTX_R 1.1×10^{-3}M cells it is about 33% of the hybridization to the wild-type sized DNA fragment in these cells which hybridizes to the same subportion of the probe; in EL4/11:MTX_R 1.1×10^{-3}M cells the corresponding figure is about 23%. This indicates that there are multiple copies of the rearranged fragment, suggesting that rearrangement occurred at an early stage in selection and that the rearranged DNA was subsequently amplified. It is therefore pertinent to ask whether the rearrangements occurred as a direct consequence of gene amplification, or whether three out of four cells in the original EL4/WILD population had one of a variety of DNA rearrangements near the DHFR gene before MTX selection was applied. While these two possibilities have not been distinguished in a direct way by examining cloned EL4/WILD cells which have not been selected for MTX resistance, two factors favour the former possibility. Firstly, although the EL4/WILD cell population used has not, so far as is known, been cloned since its origin in a C57Black mouse in 1945 (96),

the structure of the DHFR gene visualized on a Southern blot is identical to that seen in C57Black liver DNA (fig. 3.12. a-c.). If extensive DNA rearrangements had occurred in the EL4/WILD population, it would be expected to produce a more complex pattern in EL4/WILD DNA than in C57Black liver DNA. Secondly, extensive rearrangement has occurred in other amplified DNA in MTX-resistant EL4 cells, but no rearrangement in sequences which are not amplified in these cells has been found (chapter 5).

Gene amplification of a unique sequence involving a unit of less than a whole chromosome, even in the simplest models imaginable, necessarily involves some DNA rearrangement of the kind that could be visualized on a Southern blot as the change in size of a restriction fragment detected with a suitable probe; for example, multiplication of an intact block of chromosome either as an episome or as part of a chromosome would produce changes in the restriction fragments derived from sequences at either end of the amplified unit. None of the MTX-resistant cells described here (or elsewhere) contains hundreds of extra copies of a whole chromosome (section 5.1.). Thus it might seem that the a priori problem is to explain why DNA rearrangement is not observed in most cell lines, rather than why it is observed in some EL4 cell lines. However, when it has been possible to estimate the size of the amplified unit, this has been found to be in the range 500-3000 Kb (140,141,92); in contrast, the sequences detected by a DHFR cDNA probe have been estimated to be spread over 42 Kb (123) or, more recently, 32 Kb (124)

in the genome. Thus it has been thought that the block of DHFR sequences is located away from the ends of the amplified unit and therefore remains unchanged during the amplification process. This suggests one possible explanation for the rearrangement observed in the EL4 cells: that an amplification unit with one end very close to the DHFR gene has been chosen. This explanation cannot be conclusively ruled out for the EL4/8:$MTX_R^{1.1x10^{-3}M}$ cells or for the EL4/11:$MTX_R^{1.1x10^{-3}M}$ cells, but it can be excluded for the EL4/3:$MTX_R^{2.2x10^{-3}M}$ cells and thus appears unlikely for any of the EL4 cells. The EL4/3:$MTX_R^{2.2x10^{-3}M}$ cells have DNA rearrangements near both 5' and 3' ends of the gene, yet the size of the amplified unit is far larger than the 30 or 40 Kb which separates these (section 5.3.). Therefore DNA rearrangement must have occurred within the amplified unit.

During the last few years, many examples of DNA rearrangement have been detected in eukaryotic cells. These will be divided here into two categories: sequence-specific rearrangements, which involve specific DNA sequences and which may occur at a defined stage in development or seemingly at random times; and sequence non-specific rearrangements, which appear to involve any sequence that is in the right place at the right time. The best-characterized types of rearrangement fall into the first category, and include immunoglobulin genes, trypanosome surface antigen genes, copia-like sequences in Drosophila, and transposons and mating-type loci in yeast. The second category consists of those rearrangements which accompany DNA integration in

DNA-mediated gene transfer experiments. Each of these examples will be discussed briefly.

Mouse immunoglobulin gene rearrangement. At least two kinds of DNA rearrangement are involved in immunoglobulin gene expression, and have been characterized in the mouse. One is the assembly of active or abortive light chain and heavy chain genes. In the case of the light chain gene, the active configuration is produced by somatic recombination between the 3' end of one of several hundred V_L regions and the 5' end of one of 5 or so J_L regions on the same chromosome, probably with deletion of the DNA between these sites (142). Assembly of an active heavy chain gene is a similar kind of process except that there is an additional D region between the V_H and J_H regions (143). A short conserved sequence is found at the joining regions and may be involved in the recombination (143). Although in some of the myelomas studied only one of the chromosomes has undergone rearrangement and the other retains the germ-line configuration, in about half of the tumours both chromosomes have undergone rearrangement. In these cells the second rearrangement is abortive: that is, it does not lead to the production of a second functional immunoglobulin. There are multiple ways in which this has happened: recombination can occur in such a way as to produce an out-of-phase reading frame in the J region (144), or may lead to joining of the V region with a pseudo-J sequence situated between the J and C regions, thus deleting the J region (145). Other forms of abortive recombination may be found. Thus it appears that the mechanism

of recombination must allow considerable flexibility of joining points.

The second kind of rearrangement involved in immunoglobulin gene expression is the heavy chain class switch. The assembled V_H-D-J_H region is expressed at one stage of development with a $C_{H\mu}$ region to produce an IgM molecule but at a later stage with a $C_{H\gamma}$ region to produce IgG, and may be followed by a $C_{H\alpha}$ or $C_{H\varepsilon}$ to produce IgA or IgE. The mechanism proposed for such a switch again involves site-specific recombination between homologous sequences on different parts of the same chromosome with deletion of the DNA region in between (146).

Immunoglobulin gene expression has also been detected in T cells: V_H-containing receptors have been found on the cell surface although C_H regions and light chains are probably not expressed as proteins (147). Nevertheless, rearrangement of $C_{H\mu}$ region gene sequences has been detected in T cell lymphomas (148,149) and may be associated with transcription but not translation of these regions. If so, the kind of rearrangement must differ from those found in B cells since only the heavy-chain gene is involved.

Trypanosome coat protein gene rearrangement. Information on Trypanosoma brucei surface antigen gene rearrangement is more limited. Two groups have carried out the experiment of hybridizing a cDNA clone of the surface antigen to a Southern blot of genomic DNA from several strains of trypanosomes (150,151). Both groups found evidence for DNA rearrangement; in one case rearrangement of the cloned sequence only in the

trypanosome strain which was expressing that sequence (151), while in the other case rearrangement of the sequence investigated had occurred in all strains, whether or not they were expressing it (150). The first-mentioned result fits better with current ideas about expression in this system (152), but the discrepency remains to be resolved and details of the rearrangement are unknown.

Transposable mating type loci in yeast. Two loci in Saccharomyces cerevisiae, HML and HMR, contain silent copies of the mating type gene, each of which can be either a or α. Expression of the mating type gene occurrs at a third locus, MAT, and the expression has been shown to be associated with transposition of DNA from HML or HMR to MAT (153,154). The mechanism of transposition leaves an unaltered a or α gene at the HML or HMR locus while changing the mating type gene at MAT. It does not appear to involve generation of an extrachromosomal copy of a or α , but the details of the process are unknown.

Transposable elements in Drosophila. Families of repeated sequences thought to be capable of transposition have been known in Drosophila melanogaster for several years and three such families, copia, 412 and 297 have been studied in some detail (155-158). Evidence that these sequences are mobile is that in different fly strains (156) or cultured cell lines (157), different numbers of the sequences are found in different sized restriction fragments on Southern blots, or at different sites in polytene chromosomes; it is not thought that DNA rearrangement is linked with the normal developmental processes as in the examples described above.

Other evidence is consistent with mobility: members of a family are highly conserved but the different families are different. Detailed structural analysis has been carried out on copia. It is about 5 Kb long and contains perfect direct terminal repeats of 267 bp (158); at the end of this direct repeat a short imperfect inverted repeat is found. There are differences of one or two bp between the direct terminal repeats of different members of the copia family. At supposed sites of integration into the Drosophila genome a duplication of a 5 bp sequence present once in the target DNA has been found: this sequence is different at different integration sites (159). It has been suggested that the identity of the terminal repeats of a single copia element, contrasting with the difference found between elements, arises because transposition carries the information for only one of the repeats, and an excision-replication model has been proposed (158).

Transposition elements in yeast. The Ty1 element in Saccharomyces cerevisiae is present in about 35 copies per haploid genome, hybridizes to different sized restriction fragments in different strains, and has been found to hybridize to a new fragment in two out of five subclones of a yeast clone maintained independently for 30 days (160): it is therefore mobile. It is about 5.6 Kb long and is flanked by 334 bp direct repeats called δ. Insertion of Ty1 is associated with duplication of a 5 bp sequence present once before insertion (161,162); excision commonly leaves one copy of δ at the integration site, and may therefore occur

by homologous recombination (161,162). At least 30 copies per haploid genome of solo δ are found (160). The similarity with copia-like elements in Drosophila is striking; one difference is the presence of solo δ elements in yeast.

DNA rearrangement following DNA-mediated gene transfer. Although attempts to transfer genes to mammalian cells using purified DNA as a donor have been made for many years, it was only with the introduction of the system using Herpes Simplex Virus thymidine kinase (HSV-TK) gene as donor, mouse LMTK$^-$ cells as recipient, and HAT medium as the selective agent (163,164) that unequivocal success was obtained and it became possible to study the molecular events accompanying gene transfer. It was found that the donor DNA becomes integrated into high molecular weight cellular DNA in successful transformants (165,166), and more recently it has been shown by chromosome-mediated gene transfer experiments (167) and in situ hybridization (168) that there is a single major site of integration of donor DNA into a recipient cell chromosome. The aspect which is significant to the work described in this thesis is that integration, which must involve DNA rearrangement, is non-specific with respect both to the site in the donor DNA and the site in the recipient chromosome. Furthermore, DNA present in the gene transfer mixture other than the HSV-TK gene, such as the bacteriophage ϕx174 or the plasmid pBR322, is also incorporated into the TK$^+$ mouse cell DNA (166). Again, integration is not site-specific and the number of copies of the sequence which are incorporated can vary from one to several hundred in

independently arising transformants (166). Two lines of evidence suggest that such non-selected DNA is integrated into the same region as the TK gene in a particular cell line. Firstly, when selection for the loss of the TK gene is applied, part or all of the co-transfered DNA is also lost (168). Secondly, in situ hybridization to Buffalo Rat liver cell line chromosomes from cells which have incorporated both the HSV-TK gene and a plasmid containing the human growth hormone gene shows hybridization to the same chromosomal region whether the TK gene or the human growth hormone gene is used as a probe (J Jackson, personal communication). Thus the scheme that emerges for the gene transfer process is that a cell takes up many foreign DNA molecules in a non-specific fashion. These may then be partially degraded and the ends ligated together, or ligation at internal sites may occur. In either case, the result is the assembly of a large unit of foreign DNA which can be hundreds if not thousands of Kb long. Subsequently the unit may be integrated into a recipient cell chromosome. Thus the outcome is extensive and sequence non-specific DNA rearrangement.

Returning now to the DNA rearrangements which have occurred in the EL4 cells, it can be asked whether the examples listed throw any light on this process. Transposons of the Ty1 or copia type have not been identified in vertebrates, although the proviral forms of some of the retroviruses show similarities of structure (169). If the observed changes in restriction fragment size in the EL4 cells were due to the precise excision of a hypothetical

transposon situated near the DHFR gene in the EL4/WILD cells, it would be expected that the new DNA fragment would be the same size in each case. All three rearrangements were found to be different in size. The same objection can be raised to rearrangement by the insertion of a transposon at a precise site. Also, the probability of three detectable insertion events near the 3' end of the gene and none in the middle is small if insertion is random. But perhaps the strongest objection to the involvement of a transposon is that the rearrangements occurred at an early stage in amplification and have since remained stable: if transposition were frequent enough in the early stages to generate observable changes in three out of four cell lines, it should also occur at later stages and give rise to far more complex patterns of DHFR gene restriction fragments than were observed. This will be called the 'rearrangement switch-off' problem.

The DNA rearrangement involved in the yeast mating type switch is specific to this system: mating in mice is by a sexual process. DNA rearrangement in trypanosomes is too poorly characterized to serve as a useful model. Immunoglobulin gene rearrangement, however, may be relevant. EL4 cells are T-cell derived (section 2.2.4.). It is possible that the machinery for DNA rearrangement, which is switched on at some stage in normal T-cell development, remains active in the tumour cells in culture and can involve the DHFR gene. Too little is known about DNA rearrangement in T cells to decide whether this is a likely possibility. It is of interest that, in the case of myeloma lines, there is some evidence

that immunoglobulin gene rearrangement continues in the tumour cells. When B cells were isolated from blood by staining for Ig and selecting positive cells with a fluorescence activated cell sorter, it was found that the cell population retained an average of about one gene per diploid genome in the germ-line configuration (170): therefore in most cells rearrangement of immunoglobulin genes can have occurred only on one chromosome per cell. However, of thirty myeloma lines examined in one study, about half had rearrangements on both chromosomes (171). Thus immunoglobulin type gene rearrangement might be involved in DHFR gene amplification in the EL4 cells. However, the rearrangement switch-off problem also arises here if such a mechanism is invoked. A further point is that the two MTX-resistant B cell lines studied show no evidence of DNA rearrangement near the DHFR gene, nor do MTX-resistant L1210 and L5178Y cells examined by others (123). This could reflect a difference between B and T cells, or could be a matter of chance: five lymphoma lines is not a large sample.

The second kind of process which might be involved is sequence non-specific DNA rearrangement. Such rearrangement clearly can occur to any DNA which has been introduced into cultured mouse cells. Whether it can also occur in the absence of DNA-mediated gene transfer, or whether gene transfer is involved in DHFR gene amplification, is unknown. If this kind of process has taken place, it might be expected that DNA rearrangement would have involved other sequences, and it is not clear why detectable rearrangement near the

DHFR gene should be restricted to the MTX-resistant EL4 cell lines. These questions lead to a general discussion of the amplification process and are pursued in chapter 6.

4.5. CONCLUSIONS.

The conclusion from sections 4.2. and 4.3. is that it is very easy to suggest factors which may explain the complicated relationships between DHFR gene number, DHFR enzyme overproduction and MTX resistance level. A study aimed at elucidating the details of MTX resistance or DHFR gene expression should investigate these factors in detail. This thesis, however, is aimed at studying gene amplification. From this point of view, the important finding is that DHFR gene amplification has occurred in the MTX-resistant cell lines under investigation. Also important is the observation that the structure of the DHFR gene is similar to wild-type in most resistant lines, but that DNA rearrangement has accompanied amplification in a few lines.

CHAPTER 5

RESULTS PART II

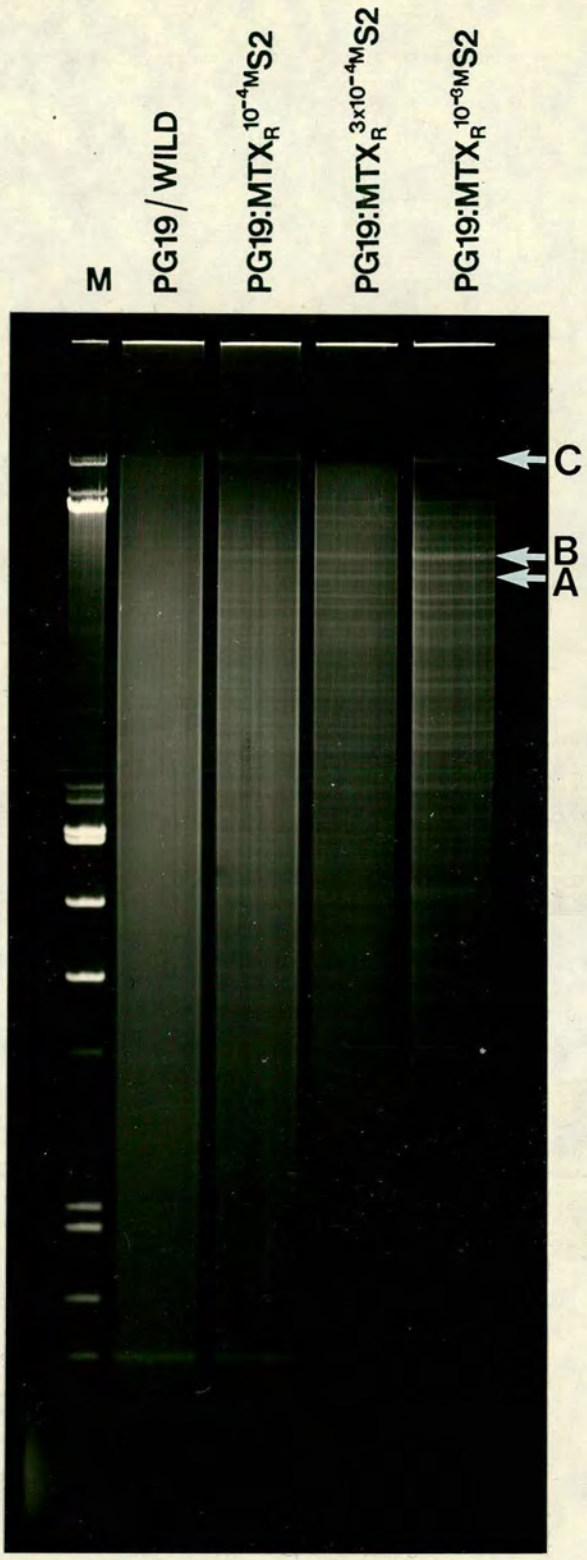


Fig. 5.1. Analysis of total nuclear DNA from wild-type and successive stages in selection of MTX-resistant PG19 cells. DNA samples were digested with Eco RI and electrophoresed in a 0.75% agarose gel. The gel was stained with ethidium bromide, illuminated with u/v lamps, and the fluorescence was photographed. M, the marker track, is a mixture of whole λ DNA and a double digest of λ DNA with Eco RI and Hind III; some feint bands are partial digestion products.

to this rule is the band marked A in the figure. In the DNA from cells resistant to 10^{-3} M and $3x10^{-4}$M MTX it is similar in intensity to the band in the same track marked B, while in the DNA from cells resistant to 10^{-4}M MTX it is very much feinter than the band B in this track. Another exception is the very high molecular weight DNA at the top of the gel (marked C); in this case, however, the apparent differences in intensity are probably due to artefactual differences in the size of the original DNA preparation and the amount of nonspecific DNA degradation during Eco RI digestion. These exceptions do not alter the general conclusion from fig.5.1., which is that DNA sequences, which are amplified in MTX-resistant cells, can be detected in total nuclear DNA, and that these sequences increase in amount but do not change much in structure during amplification. Comparable sequences have therefore been looked for in other MTX-resistant cell lines.

5.2.2. A comparison of amplified DNA bands in independently-selected cell lines

Fig 5.2.a. shows a comparison of PG19T3/WILD DNA with PG19T3:MTX_R 10^{-4}M S1 DNA and PG19T3:MTX_R 10^{-4}M S2 DNA; fig. 5.2b. shows a comparison of EL4/WILD DNA with DNA from the four resistant EL4 lines, and fig.5.2c. shows a comparison of LB1 trans/WILD DNA with LB1 trans:MTX_R $4.4x10^{-3}$M DNA. In PG19T3:MTX_R 10^{-4}M S1 DNA a few rather feint new bands can be seen; some of these are similar in size to the new bands

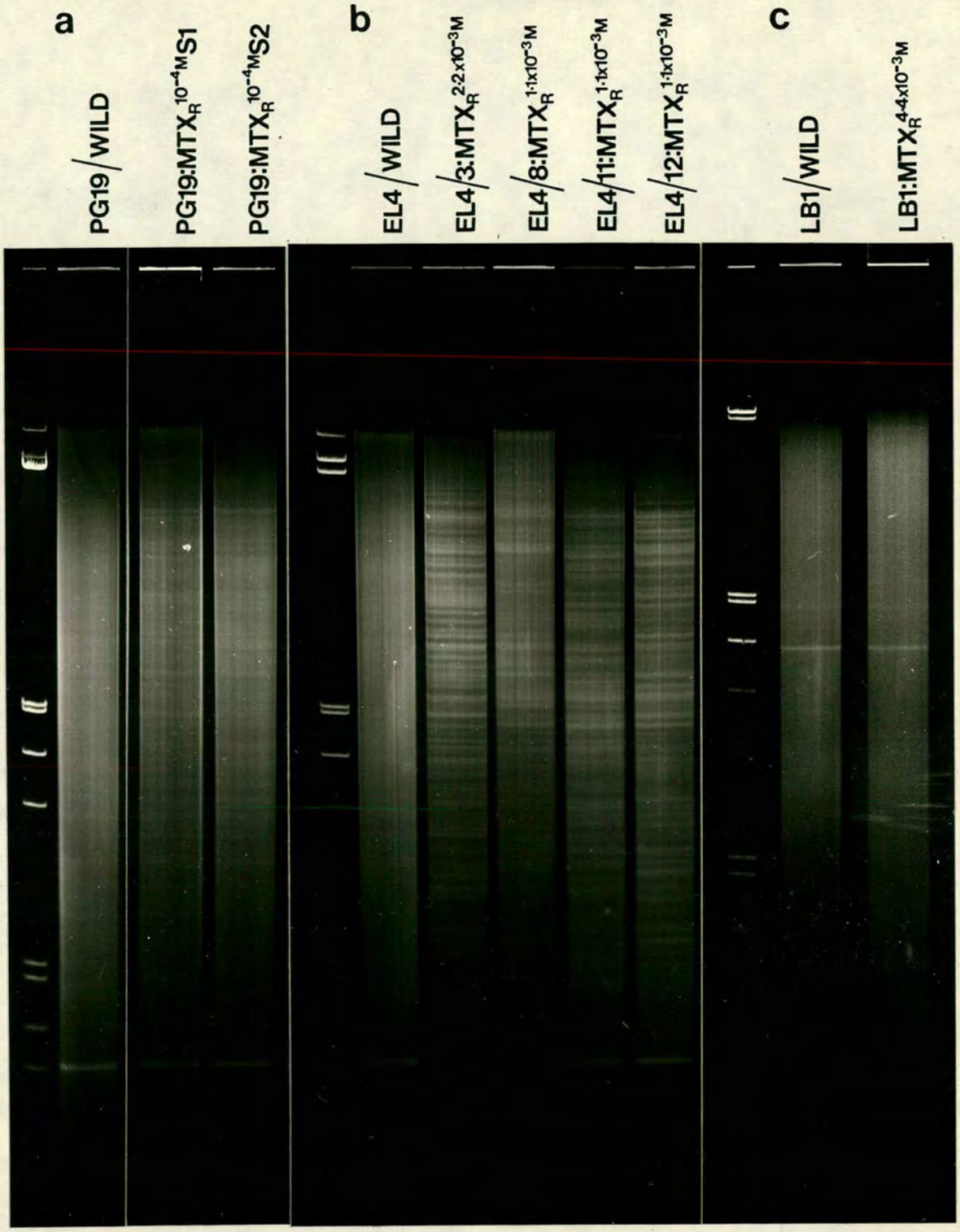


Fig. 5.2. Analysis of total nuclear DNA from wild-type and MTX-resistant cell lines.

a. PG19 DNA samples were digested with Eco RI and electrophoresed in a 0.75% agarose gel.

b. EL4 DNA samples were digested with Eco RI and electrophoresed in a 0.75% agarose gel.

c. LB1 trans DNA samples were digested with Bam HI and electrophoresed in a 1% agarose gel.

All gels were stained with ethidium bromide illuminated with u/v lamps, and the fluorescence was photographed.

in PG19T3:MTX_R 10^{-4}M S2 DNA but others are different. All four MTX-resistant EL4 cell lines show many clear new bands and again each cell line has a characteristic pattern. No new bands can be seen in the LB1 trans:MTX_R $4.4x10^{-3}$M DNA, either in fig.5.2c. or on the original negative. A visual examination of the different patterns of extra bands suggests that each pattern is unique in that it shows both the presence of endemic bands (ie. ones found only in that cell line) and a characterisitc distribution of relative intensity among those bands which are common to several cell lines. Indeed, with such complex patterns, comigration in a one-dimensional DNA gel is not good evidence that two bands in different cell lines contain the same sequence, because bands of similar size are expected to occur by chance alone.

The band patterns from EL4 cells have been analysed in more detail. In this experiment Bam HI digests were used, and only the central portion of each gel track was analysed. This limitation was applied because the background subtraction method employed only separated background from signal if the signal was more variable than the background; such was not the case at the top and bottom of the gel track. The results are shown in figs.5.3. to 5.7. In each of figs.5.3. to 5.6., part A shows a photograph of the appropriate part of the gel track, part B shows the COSMOS scan of it, part C shows the calculated background and part D shows the total scan after background has been subtracted from it. Thus each peak in part D represents a band in the DNA, displayed with the continuum of unresolved DNA fragments removed. In wild-type DNA two peaks can be

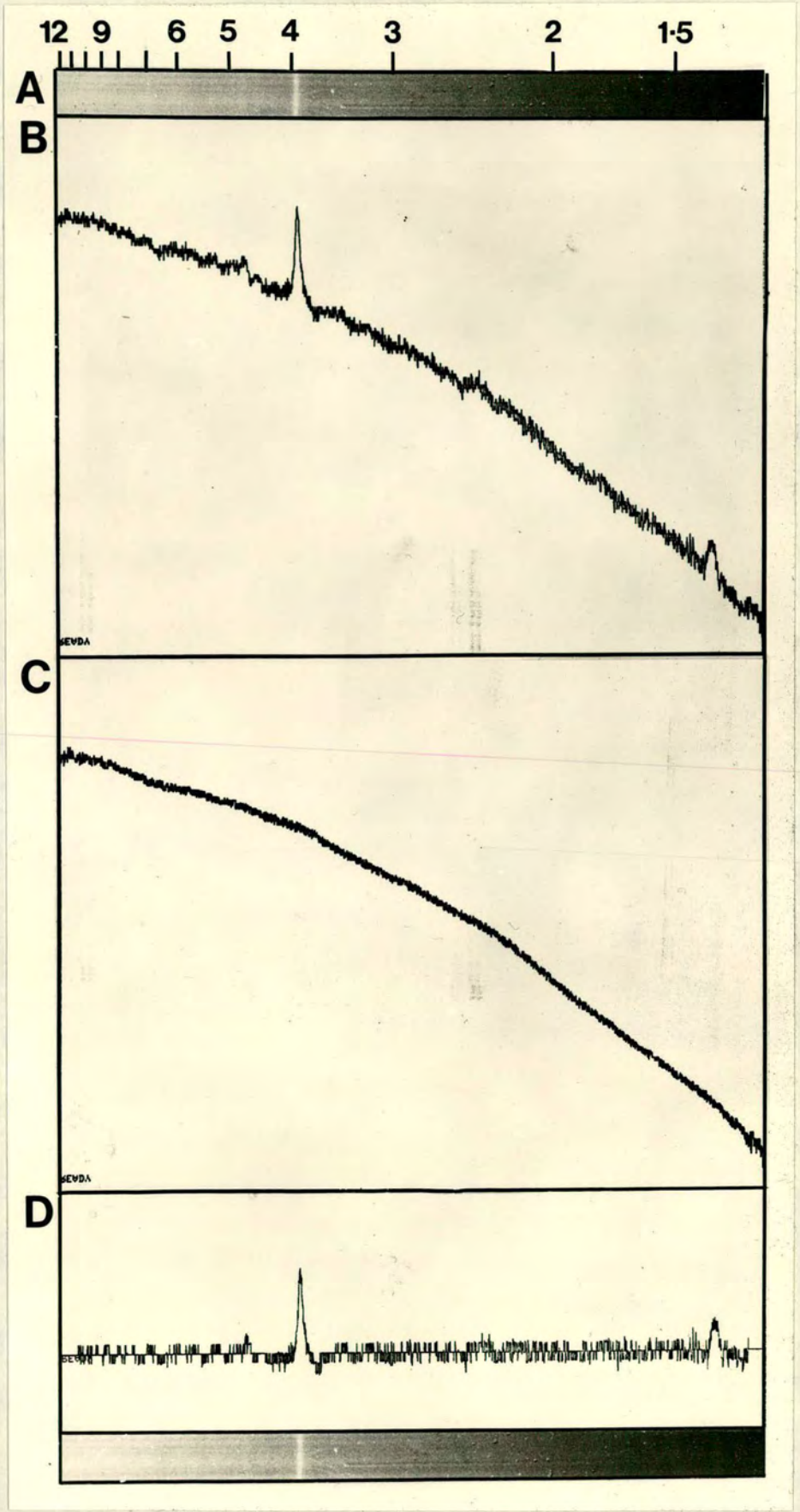


Fig. 5.3. Analysis of Bam HI digest of EL4/WILD DNA.

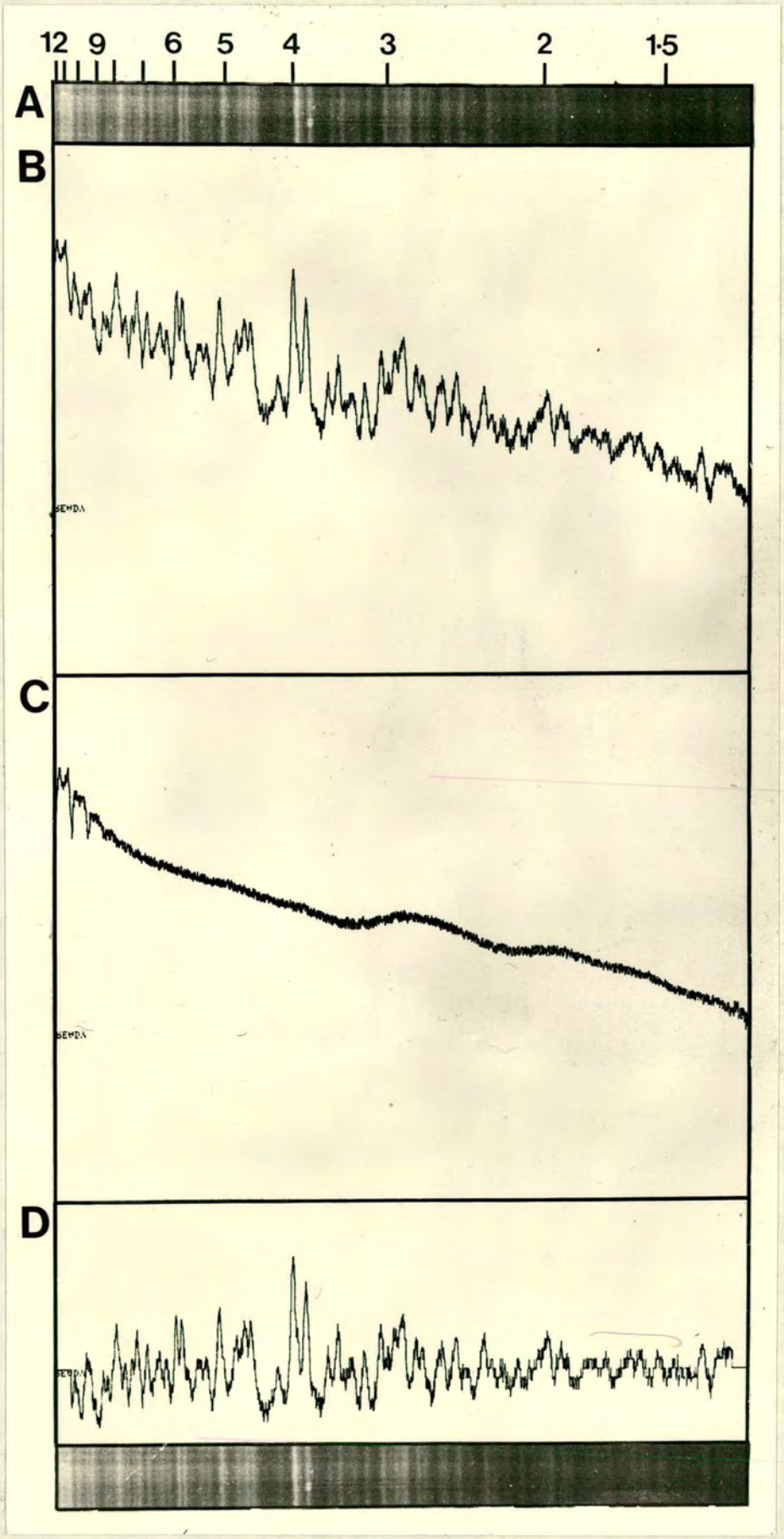


Fig. 5.4. Analysis of Bam HI digest of EL4/3:MTX_R 2.2×10^{-3}M DNA.

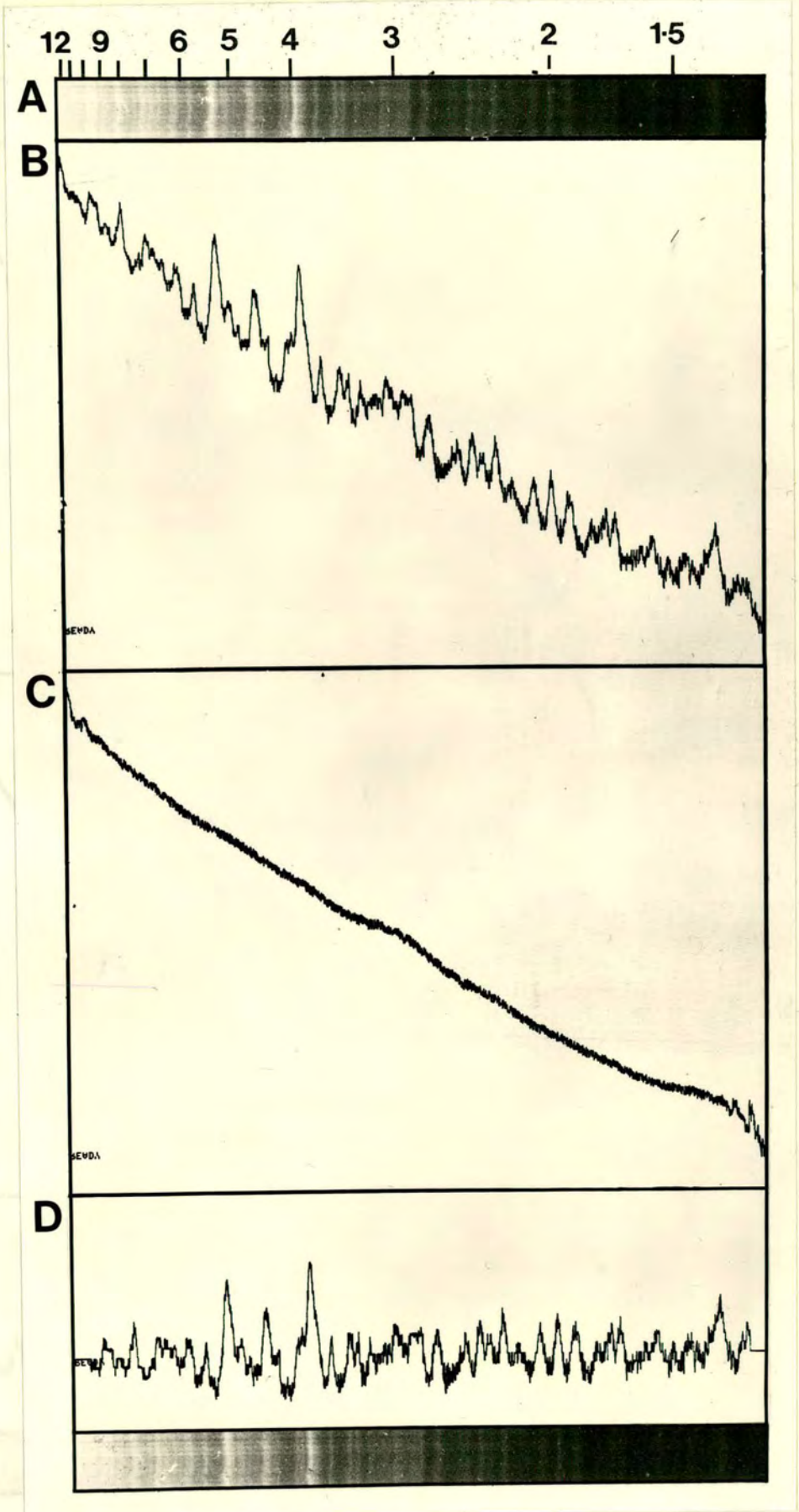


Fig. 5.5. Analysis of Bam HI digest of EL4/8:MTX$_R$ 1.1×10^{-3} M DNA.

Figs. 5.3. to 5.6. Legend.

A. Nuclear DNA was digested with Bam HI and electrophoresed in a 1% agarose gel. The gel was stained with ethidium bromide, illuminated with u/v lamps, and the fluorescence was photographed. The portion of the gel track analysed in detail is shown; the top and bottom are omitted. The numbers above A are the approximate sizes of the DNA molecules in Kb.

B. COSMOS scan of the negative from which A was prepared: the 'raw data'.

C. Calculated background to B.

D. C subtracted from B: the 'background subtracted data'.

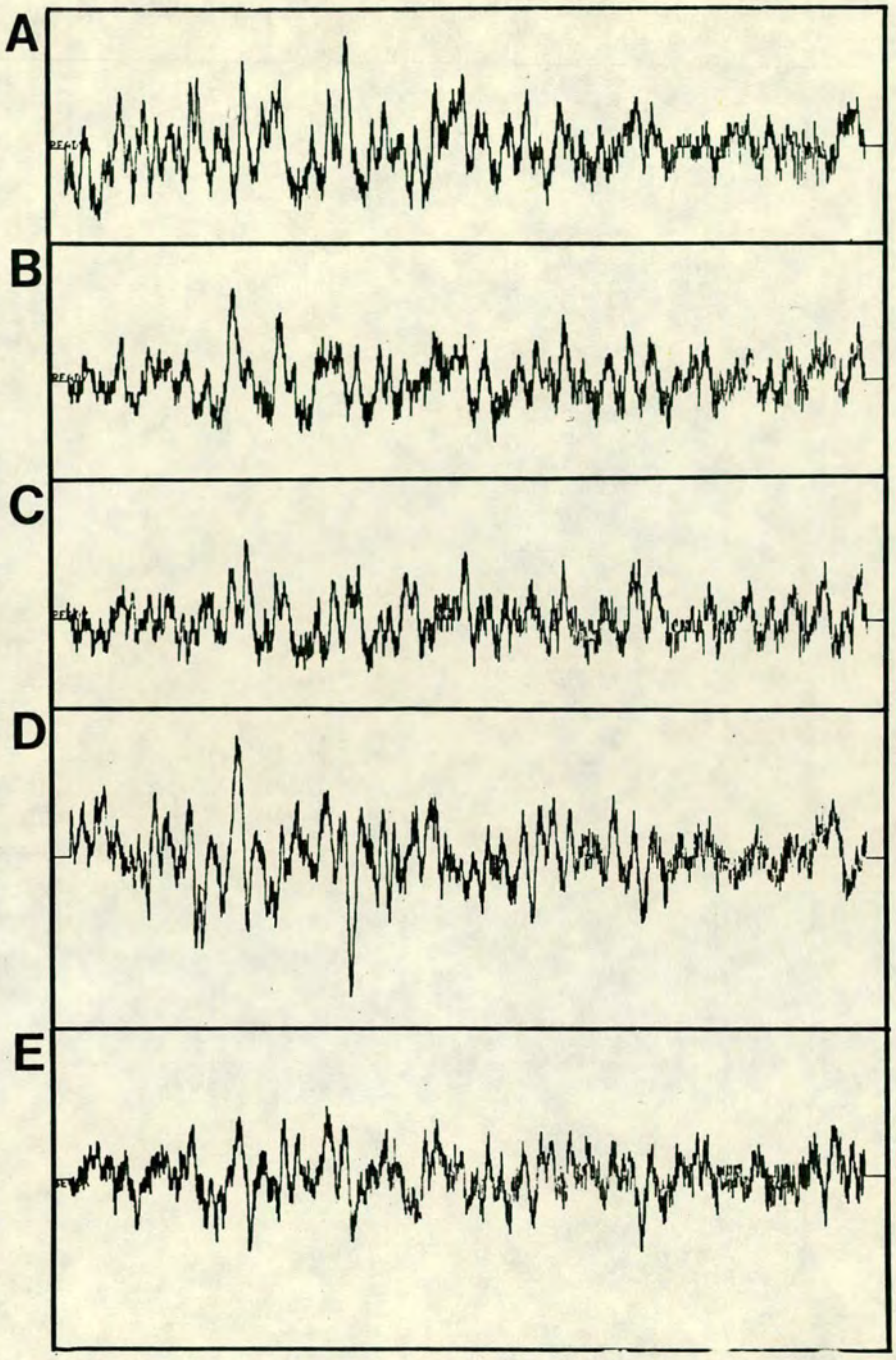


Fig. 5.7. Analysis of the amplified DNA in Bam HI digests of MTX-resistant EL4 cell nuclear DNA.

A.- C. Subtraction of the EL4/WILD peaks (fig. 5.3.D.) from the MTX-resistant EL4 peaks (figs. 5.4.D., 5.5.D. and 5.6.D.) to show the amplified DNA pattern.

A. EL4/3:$MTX_R^{2.2\times10^{-3}M}$ - EL4/WILD.

B. EL4/8:$MTX_R^{1.1\times10^{-3}M}$ - EL4/WILD.

C. EL4/12:$MTX_R^{1.1\times10^{-3}M}$ - EL4/WILD.

D.- E. Comparison of the amplified DNA patterns.

D. EL4/8:$MTX_R^{1.1\times10^{-3}M}$ - EL4/3:$MTX_R^{2.2\times10^{-3}M}$.

E. EL4/8:$MTX_R^{1.1\times10^{-3}M}$ - EL4/12:$MTX_R^{1.1\times10^{-3}M}$.

seen (fig.5.3.d.) corresponding to repeated sequences in the normal mouse genome. In each of the DNA samples from MTX-resistant cells, these two peaks can be seen, but in addition, many new peaks corresponding to amplified sequences are visible (figs. 5.4.D., 5.5.D., 5.6.D.). Thus the technique developed here allows a clearer view of the amplified DNA sequences than can be obtained from looking at the gel photograph alone.

It does more than this, however; it enables extensive manipulation of the data to be carried out. This has been done in two stages. First, the EL4/WILD peaks have been subtracted from each of the MTX-resistant DNA patterns and the results displayed in fig 5.7., A to C. Secondly, these resulting patterns, each of which represents only amplified DNA, have been compared with one another. This has been done by subtracting from the EL4/8:$MTX_R^{1.1\times10^{-3}M}$ pattern either the EL4/3:$MTX_R^{2.2\times10^{-3}M}$ pattern or the EL4/12:$MTX_R^{1.1\times10^{-3}M}$ pattern. The results are shown in fig.5.7.D. and E. In each case, the observation that there are both positive and negative peaks in the difference pattern shows that the two DNA samples compared each have sequences that the other lacks; and the lack of similarity between the two difference patterns indicates that all three DNA samples differ. This analysis therefore confirms and extends the conclusion reached from visual inspection of the gel patterns: that different sequences have been amplified in each cell.

5.2.3. A comparison of amplified DNA bands in clones of the MTX-resistant cell populations

With the exception of the PG19T3:MTX$_R$ 10^{-4}M S1 cell DNA, all of the DNA samples used in subsections 5.3.1 and 5.3.2. were derived from cell populations which had not been cloned since the initial selection stage. It is therefore unclear to what extent the complex patterns found reflect heterogeneity between cells in the populations or between amplified units within a cell, and to what extent they reflect the array of sequences found within a large amplification unit.

Attempts to clone the MTX-resistant EL4 cells were unsuccessful, using either soft agar or a microwell plate. It seems that their plating efficiency at low density is extremely low. However, the MTX-resistant PG19 cells have been successfully cloned, and fig.5.8. shows Eco RI digests of DNA from some of the resulting clones. In fig.5.8a. two separate clones resistant to 10^{-4}M MTX which were derived from the PG19T3:MTX$_R$ $3x10^{-5}$M S1 population are compared, and in fig.5.8b. four subclones of one of these, PG19T3:MTX$_R$ 10^{-4}M S1 clone 15, are compared. It can be seen that the patterns of amplified DNA bands are very similar in all clones, and any slight differences are no more than would be expected from different preparations of PG19T3:MTX$_R$ 10^{-3}M S2 DNA made from the uncloned population. The first preparation was made as soon as about 10^7 cells resistant to 10^{-3}M MTX had been obtained; the second was made after the population had been growing continuously under selection for about six weeks (30 to 40 generations). Fig 5.8c. also shows three separate clones of population: clones 4,7 and 8.

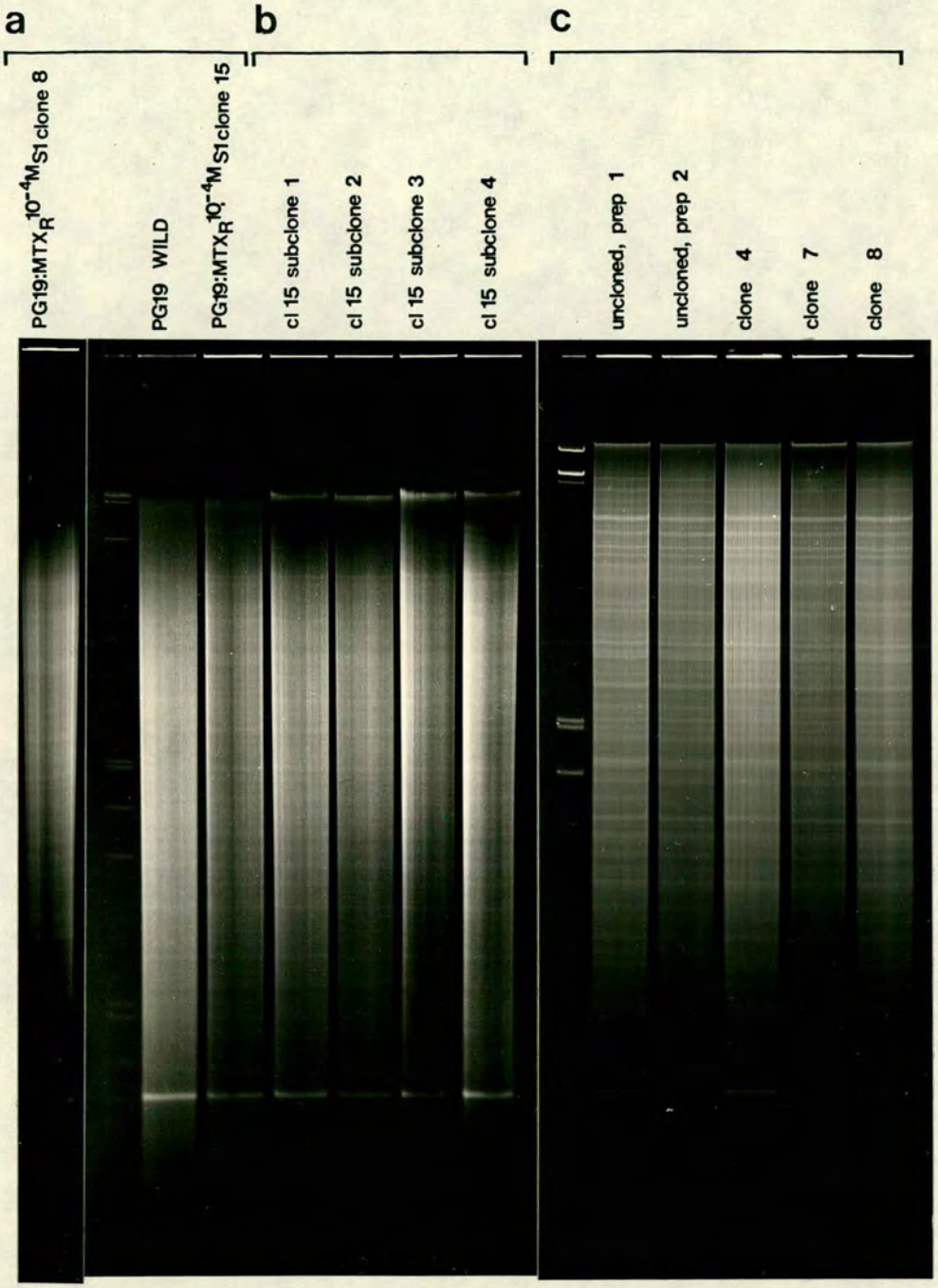


Fig. 5.8. Analysis of total nuclear DNA from cloned MTX-resistant PG19 cells. All DNA samples were digested with Eco RI and electrophoresed in a 0.75% agarose gel. The gels were stained with ethidium bromide, illuminated with u/v lamps, and the fluorescence was photographed.

a. Clones of PG19 S1 cells resistant to 10^{-4}M MTX.

b. Subclones of PG19T3:MTX$_R$ 10^{-4}M S1 clone 15.

c. Two different preparations of DNA from the PG19T3:MTX$_R$ 10^{-3}M S2 population and three cloned derivatives of this cell line.

The patterns of amplified DNA bands in all five DNA samples are very similar. Thus the results in fig.5.8. show that most, if not all, cells in the population contain the same amplified DNA sequences.

Fig 5.8. does not, however, indicate whether there is any heterogeneity between amplified units within a cell. It is difficult to think of an experiment in which this problem could be investigated. Nevertheless, purely by chance, a biological system arose which made it possible to characterize the DNA of what is probably a single amplified unit. Over a period of about two weeks during the summer of 1980, it was observed that in one subculture of EL4/8:$MTX_R^{1.1x10^{-3}M}$ cells maintained in 1.1×10^{-3}M MTX, cells containing the dms and ring chromosomes described in section 5.1. were replaced by cells lacking these features but containing three new marker chromosomes. Examination of chromosome preparations made during this period suggested that the new marker chromosomes had the following origin (C.J.Bostock, personal communication). A dm, possibly a submicroscopic ring itself, gave rise to a microscopically discernible acentric ring which, probably by crossing over an odd number of times, increased in size. Very large ring chromosomes generated in this way were unstable and broke to form linear chromosome fragments, and one such fragment gained a centromere. The cells possessing the resulting centric chromosome had a selective advantage over other cells in the population because they carried the amplified copies of the DHFR gene essential

for survival in a stable form, whereas the other cells carried the DHFR gene in an unstable form (section5.5.). These cells outgrew the rest of the population, and came to replace them entirely. If this interpretation of events is correct, the process led to the DNA from a single dm becoming the major form of amplified DNA in the population. Thus a comparison of the amplified DNA in the cells containing dms with that in the cells containing the centric marker chromosomes will allow the sequences in the population of amplified units to be compared with the sequences in a single dm, and hence probably those in a single (or at the most a few) amplified unit(s). Fig.5.9a. shows such a comparison: the sequences are very similar. Therefore most, if not all, amplified units in the parent EL4/8:$MTX_R^{1.1\times10^{-3}M}$ cell line have the same structure. Fig.5.9b. re-enforces this conclusion: the DHFR sequences in the two EL4/8 DNA samples illustrated in fig 5.9a. have been visualized. In this cell line, DNA rearrangement has led to the appearance of a new restriction fragment containing sequences from the 3' end of the DHFR gene (chapters 3 and 4). This new fragment (arrowed) is present in approximately the same proportion in both DNA samples.

Thus the conclusion reached from section 5.2. is that the DNA sequences within the amplified units in a single cell line are derived from the same parental wild-type cell. The structure of the amplified unit is stable during growth for many generations, and when selection

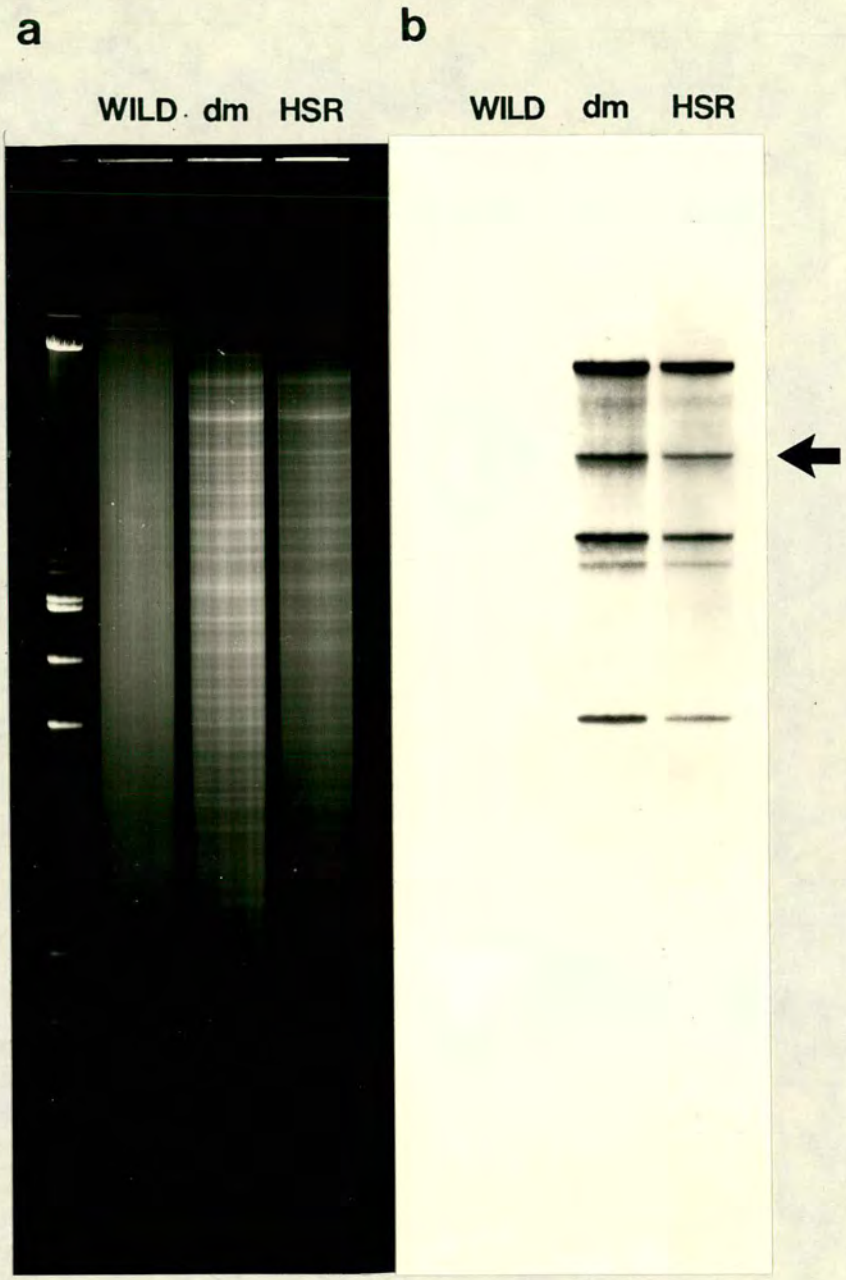


Fig. 5.9. Comparison of EL4/8:MTX$_R^{1.1x10^{-3}M}$ DNA from cells with dms and cells with HSRs.

a. Total nuclear DNA was digested with Eco RI and electrophoresed in a 0.75% agarose gel. The gel was stained with ethidium bromide, illumunated with u/v lamps, and the fluorescence was photographed.

b. The gel shown in a was transfered to nitrocellulose, the filter was hybridized with ^{32}P-labelled pDHFR 11 and autoradiographed. The arrow indicates the band which has been generated by DNA rearrangement.

for increased numbers of DHFR genes is applied, the amplified DNA increases in whole units without much change in structure.

5.3. ESTIMATION OF THE SIZE OF THE AMPLIFIED UNIT

Since total amplified DNA has been visualised using the two different techniques of metaphase chromosome spreads and DNA gel electrophoresis, the amount of amplified DNA, as a proportion of total DNA, can be estimated in two ways. Thus, since the approximate number of amplified DHFR genes has been determined, two independent estimates of the size of the amplified unit can be obtained.

Estimation of the amount of additional chromosomal material present in MTX-resistant cell metaphase spreads is complicated by chromosomal heterogeneity between cells. The simplest case, because the chromosomes are the most homogeneous, is the EL4/11:$MTX_R^{1.1x10^{-3}M}$ cell line. Here there are on average seven normal-sized homogeneously staining chromosomes in addition to the EL4/WILD complement of 40 (section 5.1.). These extra chromosomes must contain at least $7/40 \times 6 \times 10^9$ bp = 1.05×10^9 of DNA. Since there are about 700 copies of the DHFR gene per haploid genome (table3.2.), the size of the amplified unit is about 7.5×10^5 bp.

EL4/8:$MTX_R^{1.1x10^{-3}M}$ cells contain on average 64 dms in addition to the 40 EL4/WILD chromosomes. If it is assumed that a dm in these cells contains on average between one tenth and one twentieth of the DNA of a normal

mouse chromosome, this will correspond to about 5×10^8 bp of DNA. Since these cells contain about 700-1300 copies of the DHFR gene per haploid genome (table 3.2.), estimates for the size of the amplified unit vary from about 2×10^5bp to 8×10^5bp.

Estimates can be made in similar ways for EL4/3 and EL4/12 cells, and the figures are of the same order. However, it is very difficult to estimate the amount of chromosomal material in the dms of the PG19T3:MTX_R 10^{-3}M S2 cells because they are so small. The size of the amplified unit estimated from the amount of additional chromosomal material will tend to be an underestimate, because not all copies of the amplified unit are present in recognizable chromosomal forms (see section 5.5.). Consequently, estimates obtained in this way must be regarded as imprecise

In principle, DNA gel electrophoresis can give a more accurate figure, since the sizes of bands resulting from the amplified DNA can be measured accurately by comparing their rates of migration with those of standards, and the measurements added together to give the total. In practice, however, this accuracy cannot be achieved because of two major factors. First, it is difficult to distinguish every band in complex patterns such as those seen in fig s. 5.1. and 5.7. Second, not all sequences which are amplified will give rise to bands visible on these gels. For example, under the conditions used, a sequence which is present in 600 copies per haploid genome would have to be approximately 10Kb long in order to be detected. The feint bands

which are larger than this size may thus be derived from sequences which are present once per amplified unit. Intense large bands and all small bands must, however, be derived from sequences which are present more than once per amplified unit, or from the chance co-migration of fragments from several sequences present once per amplified unit. For these reasons, again only a minimum size for the amplified DNA can be obtained. About 50 to 100 bands of amplified DNA can be counted in the MTX-resistant EL4 lines and the PG19S2 cells; if an average size of 5Kb is assumed for the bands, this corresponds to 2.5×10^5bp to 5×10^5bp per amplified unit. Because each band has only been counted once, and because many bands must have been missed, this figure must be multiplied by at least a factor of two or three, and possibly by a factor of ten, to obtain the true size of the amplified unit.

In conclusion, the size of the amplified unit can only be estimated very approximately in any cell line by either method, but the minimum size estimate is about 2×10^5 bp and the real size may be nearer 2×10^6bp.

5.4. BLOT HYBRIDIZATION EXPERIMENTS REVEAL AMPLIFICATION OF SPECIFIC SEQUENCES

It is clear that, in MTX-resistant PG19 and EL4 cells described here, as in other cell lines (140, 92), the amplified unit is much larger than the stretch of DNA required to code for DHFR. This amplified DNA has been characterized *in toto* in sections 5.1. and 5.2.; in this section specific sequences which may occur in it are investigated.

5.4.1. Hybridization of transcribed sequences

cDNA has been synthesized from total sensitive or resistant cell RNA primed with oligo-dT and used as a probe against Southern blots of sensitive and resistant cell DNA. Fig.5.10a. shows an experiment where the cDNA probes were from PG19T3/WILD and PG19T3:MTX_R 10^{-4}M S1 RNA, and the DNA attatched to the filter was from PG19T3/WILD, PG 19T3:MTX_R 10^{-4}M S1 or PG19T3:MTX_R 10^{-4}M S2 cells. It is expected that the cDNA synthesized from wild-type cells will be a complex mixture of species, and that the genomic DNA fragments from which they have been transcribed will fall into many size classes after digestion with a restriction enzyme; consequently the result of hybridization will be a smear. This is what is seen in all tracks (fig. 5.10.) although some bands can be seen superimposed upon this background in some of the tracks. Two ways in which such bands could be produced can be suggested: they could represent species which are particularly abundant in the mRNA, or they could be sequences which are repeated in the genome. Under the conditions of hybridization, it is likely that only sequences which are repeated in the genomic DNA will give rise to bands. The expected sizes of DHFR genomic DNA fragments are known (section3.7.) and also which fragments hybridize to the 3' end of the DHFR cDNA (fig.3.19.). It can be seen that bands at the positions of the 3' DHFR fragments show up when MTX-resistant cell genomic DNA is probed with MTX-resistant cell cDNA. The cDNA synthesized was largely a few

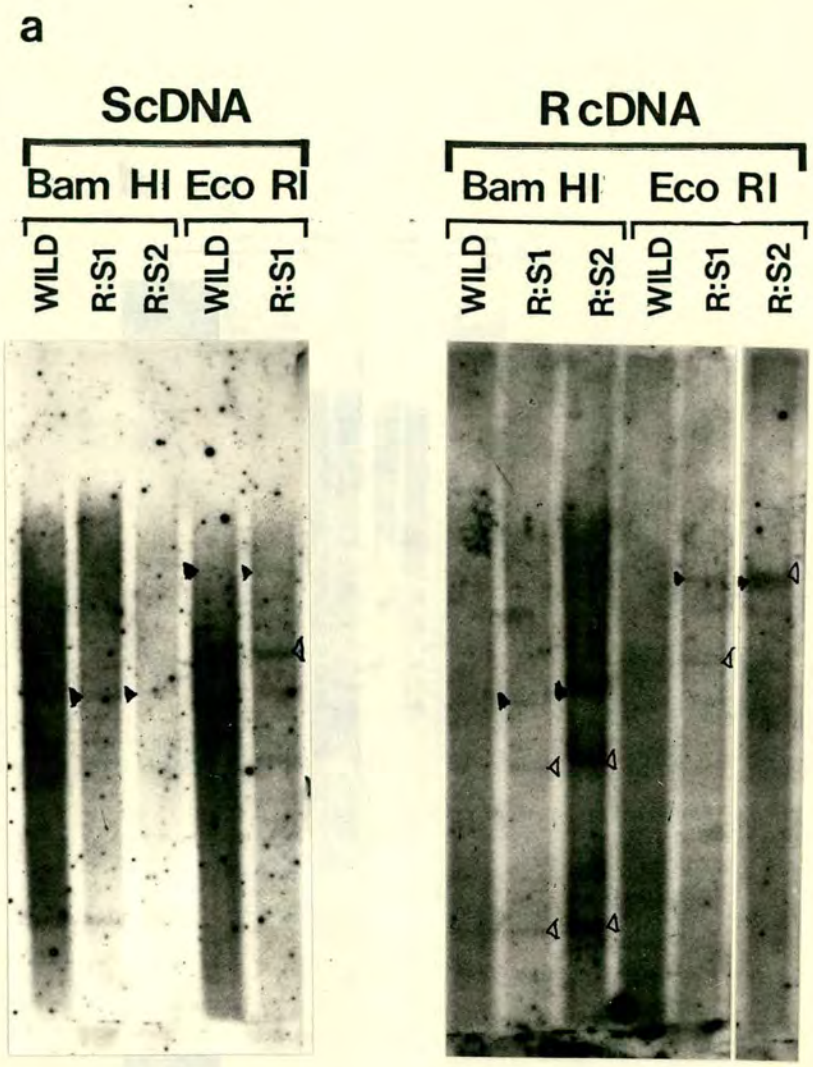


Fig. 5.10. Hybridization of wild-type and MTX-resistant cell DNA with cDNA.

a. PG19. Nuclear DNA from PG19/WILD (WILD), PG19T3:MTX_R 10^{-4}M S1 (R:S1), or PG19T3:MTX_R 10^{-4}M S2 (R:S2) cells was digested with either Bam HI or Eco RI as indicated, and electrophoresed in a 0.5% agarose gel. After transfer to nitrocellulose, filters were hybridized either with ^{32}P-labelled cDNA transcribed from PG19/WILD RNA (ScDNA), or with ^{32}P-labelled cDNA transcribed from PG19T3:MTX_R 10^{-4}M S1 RNA (RcDNA), and autoradiographed.

b

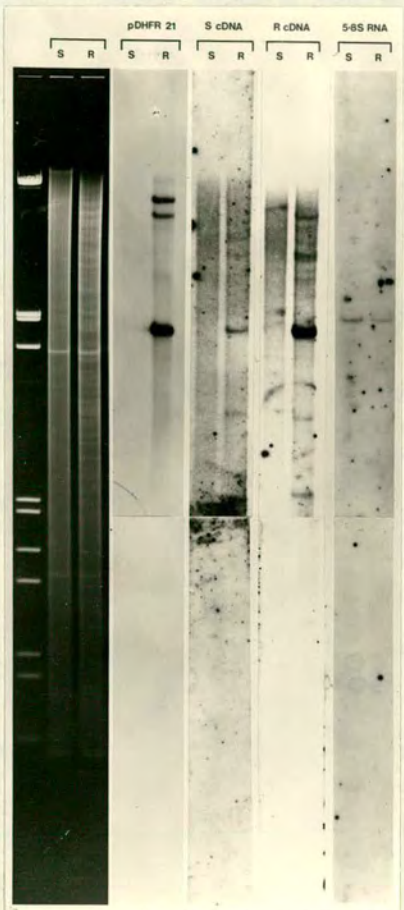


Fig. 5.10. (continued).

b. EL4. Nuclear DNA from EL4/WILD (S) or EL4/3:$MTX_R^{2.2x10^{-3}M}$ (R) cells was digested with Bam HI and electrophoresed in a 1% agarose gel. After transfer to nitrocellulose, filter strips were hybridized with the Pst I - excised insert from pDHFR 21 labelled with ^{125}I by nick translation (pDHFR 21), or with ^{32}P-labelled cDNA transcribed from EL4/WILD RNA (ScDNA), or with ^{32}P-labelled cDNA transcribed from EL4/3:$MTX_R^{2.2x10^{-4}M}$ RNA (RcDNA), or with ^{32}P end-labelled mouse 5.8S RNA (5.8S RNA) and autoradiographed. The hybridization with 5.8S RNA was carried out by E. Lund.

hundred nucleotides long (data not shown) and therefore lacks sequences from the 5' ends of most mRNA s; for this reason other DHFR genomic DNA fragments are not seen. No DHFR bands can be seen in PG19T3/WILD DNA probed with MTX-resistant cell cDNA, suggesting that even an abundant cDNA species does not detect a unique gene under these conditions. In MTX-resistant cell genomic DNA probed with wild-type cDNA, the 3' DHFR gene fragments can again be seen, although less distinctly than when resistant cell cDNA was used; again these bands cannot be seen in wild-type genomic DNA. Thus even in wild-type cells the low amount of DHFR mRNA (fig.3.9.) is sufficient to generate enough cDNA to visualise genomic DHFR bands in MTX-resistant cell DNA: this shows that a genomic sequence which is present in multiple copies can be detected in this experiment even if its transcripts are not very abundant. Fig.5.10b. shows that similar bands can be detected in EL4 cells.

In both PG19 and EL4 cells, bands which do not correspond to DHFR fragments can also be seen. In Bam HI digests of all PG19 DNA samples there is a band at about 2Kb, which must represent a sequence repeated in mouse DNA which is also complementary to transcribed sequences. This band is clearer in the DNA from the MTX-resistant cell lines; therefore some copies of it must be amplified. No other discrete bands can be seen in the PG19T3/WILD DNA digested with either Bam HI or Eco RI and probed with cDNA from either sensitive or resistant cells. In Bam HI

digests of both MTX-resistant PG19 cell lines, there is a band at about 3½ Kb which must represent a sequence which has been amplified in both these cell lines. In the Eco RI digest of PG19T3:MTX_R 10^{-4}M S1 DNA there is a band at about 8 Kb, and in the Eco RI digest of PG19T3:MTX_R 10^{-4}M S2 DNA there is a band just above the DHFR band in this track; both of these must represent sequences which have been amplified in only one cell line. Similarly, in the EL4/3:MTX_R $2.2x10^{-3}$M cell DNA, bands at about 3.0, 2.1 and a feint one at about 1 Kb can be seen representing transcribed sequences amplified in this cell line.

Thus hybridization with cDNA probes shows that in each MTX-resistant cell line there is DNA in the amplified unit which is complementary to transcribed sequences of both sensitive and resistant cells, and that each of the cell lines examined has sequences of this kind which are unique to it. However, it should be noted that these RNA species do not give rise to increased amounts of polypeptides (fig s.3.4. and 3.5.).

5.4.2. Hybridization of specific probes

"Satellite" DNA is the major repeated sequence in the mouse and makes up about 10% of the genome. Originally defined by its distinctive density in caesium chloride density gradients (174), a large part of it consists of tandem repeats of variants of a 240 bp sequence, which after digestion with enzymes such as Pst I, Hae III or

Taq I and separation according to size in an agarose gel, generate a 'ladder' with "rungs" differing in size by 240 bp. Fig.5.11. shows that such patterns are found in EL4 cell DNA. Since satellite DNA is so abundant it might be expected on the basis of chance that changes in the genome will involve it; but equally it may be difficult to detect such changes against the enormous background of unaltered satellite DNA. No significant differences between wild-type and MTX-resistant cell DNA can be seen in fig.5.11. Fig 5.12. shows DNA digested with Bam HI, which cuts satellite DNA infrequently (T.H.N.Ellis, PhD thesis, University of Edinburgh, 1979). In all tracks a background smear of unresolved fragments is seen, and superimposed upon this are a few discrete bands. These are different in wild-type and MTX-resistant cell DNA samples, indicating either that a few previously undetected Bam HI repeats have been amplified or that rearrangement has generated new fragments.

Few cloned probes were available when this work was carried out, but a fragment of the mouse genomic ribosomal repeat, rabbit α and β globin cDNA clones, and a sea urchin actin genomic clone have been used. No significant difference between sensitive and resistant cell DNA were seen when actin, α globin or ribosomal DNA were used as probes, thus showing that sequences which have homology with these probes are not amplified or rearranged. The result obtained with the ribosomal probe is shown in fig. 5.13.: it is significant because it shows that the results demonstrating amplification of transcribed sequences described

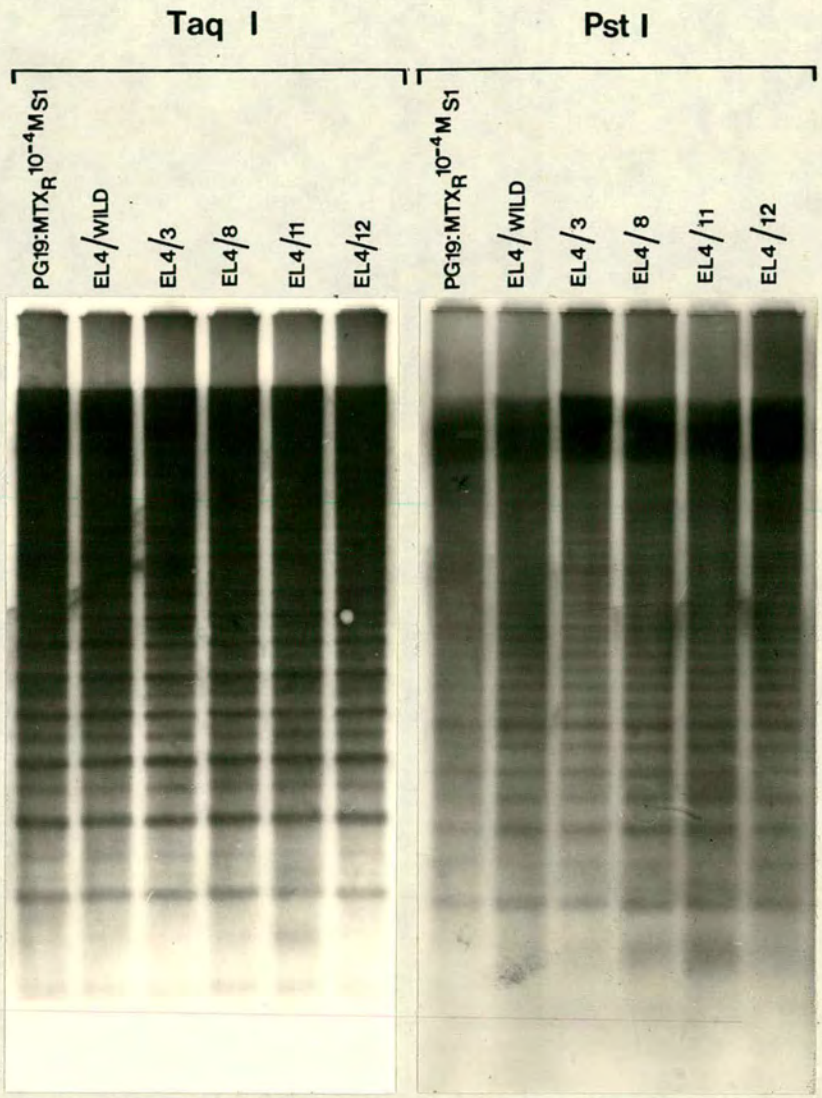


Fig. 5.11. Analysis of satellite DNA in wild-type and MTX-resistant cells. DNA samples were digested with Taq I or Pst I and electrophoresed in a 1.5% agarose gel. The DNA was transfered to nitrocellulose, and the filter was hybridized with ^{32}P-labelled mouse satellite DNA and autoradiographed. DNA samples are identified at the top of each track.

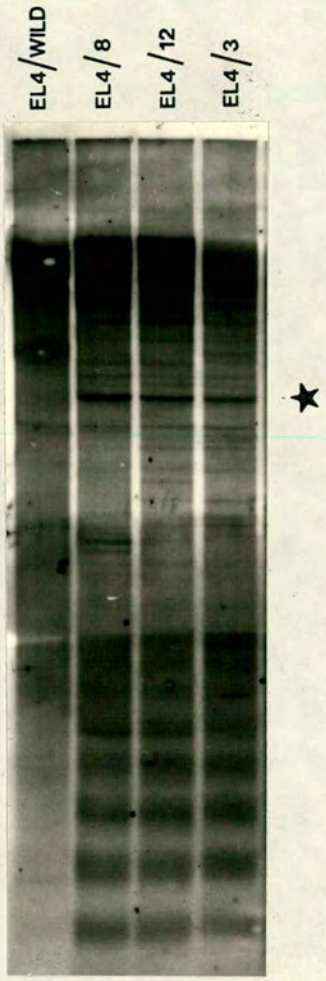


Fig. 5.12. Analysis of satellite DNA in Bam HI digests of wild-type and MTX-resistant EL4 cells. DNA samples were digested with Bam HI and electrophoresed in a 1% agarose gel. The DNA was transfered to nitrocellulose, and the filter was hybridized with ^{32}P-labelled mouse satellite DNA and autoradiographed. DNA samples are identified at the top of each track. The band marked with a star (★) derives from residual hybridization to DHFR from a previous experiment; the broad bands near the bottom of the gel are a 'nucleosomal ladder' due to degredation of the DNA samples; other bands are satellite DNA.

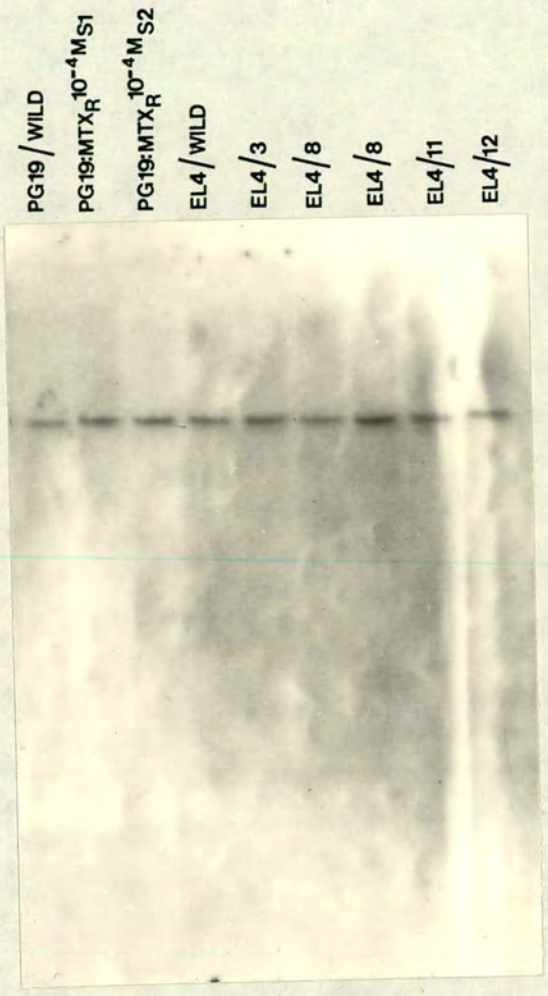


Fig. 5.13. Analysis of ribosomal DNA in wild-type and MTX-resistant cells. DNA samples were digested with Eco RI and electrophoresed in a 0.75% agarose gel. The DNA was transfered to nitrocellulose and the filter was hybridized with a ^{32}P-labelled λgtWES.rDNA clone (10f) containing a 6.6 Kb insert of mouse ribosomal DNA and autoradiographed. A single band of approximately 6.6 Kb and approximately equal intensity can be seen in each track.

in section 5.4.1. are not due to contamination of the cDNA preparation with ribosomal sequences.

The result obtained when the rabbit β globin cDNA plasmid was used as a probe are shown in fig.5.14. It can be seen that there is an extra band in the EL4/3:$MTX_R^{2.2x10^{-3}M}$ DNA track which is not present in DNA from other cell lines. Its intensity in the EL4/3 track is not much greater than that expected for a single copy of the β globin gene; therefore it cannot result from the presence of one intact β globin gene in each amplified unit. Two explanations for the band can be suggested: it could represent a structurally rearranged β globin gene or homologous sequence which is present in only a few copies per cell; alternatively it could represent a non-rearranged sequence present in many copies per cell, perhaps once per amplified unit, but which has only a small degree of homology with the probe. It is known that many such sequences - the foetal, embryonic and pseudo- β-globin genes, which cross-hybridize with rabbit β globin cDNA - exist in the mouse genome (175). These explanations have not been distinguished experimentally, although the latter would be the one predicted from the homogeneity of the sequences in the amplified units of a single cell line (section 5.2.). Additional,very feint, bands could be seen on the original autoradiograph, and their positions are marked by arrows in fig.5.14.

While it is not possible to decide whether amplification of the authentic mouseβglobin genes has occurred, these

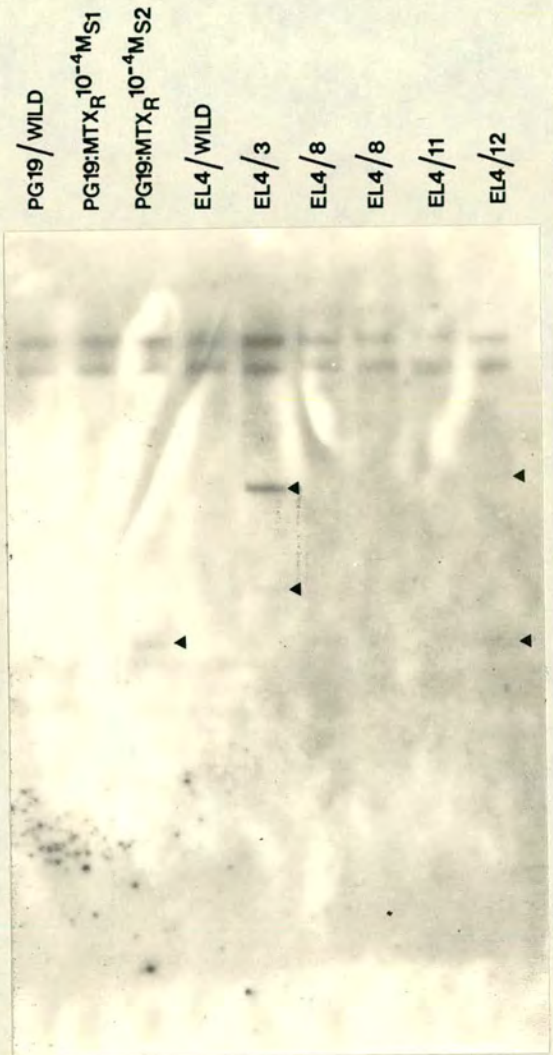


Fig. 5.14. Analysis of β-globin-like sequences in wild-type and MTX-resistant cells. DNA samples were digested with Eco RI and electrophoresed in a 0.75% agarose gel. The DNA was transfered to nitrocellulose, and the filter was hybridized with ^{32}P-labelled pβG1 and autoradiographed. pβG1 contains an insert of rabbit β-globin cDNA (107). Two bands, probably corresponding to the β-major and β-minor globin genes, are seen in each track. In addition, a smaller band is clearly seen in the EL4/3:MTX$_R$ 2.2×10^{-3}M DNA track. This and some other feint bands are arrowed (▶).

results showing amplification and/or rearrangement of a sequence which cross-hybridizes with rabbit β globin cDNA, and the results described earlier in this section, re-enforce the conclusion that extensive DNA alteration has taken place during the development of resistance to MTX in these cells, and that this is different in different cell lines.

5.5. SOME AMPLIFIED DNA IS LOCATED ON dms

In the earlier sections of this chapter, DNA which has been amplified in the MTX-resistant cells was characterized, either directly by staining with ethidium bromide, or indirectly using specific radioactively-labelled sequences as probes, and the chromosomal changes which have occurred were described. While the two kinds of change are associated in that they occur within the same cells, it has not been shown whether they are physically linked: that is, whether the abnormal chromosomes are the carriers of the amplified DNA visualized by the other techniques. This question is investigated in this section.

MTX resistance in the EL4/8:$MTX_R^{1.1\times10^{-3}M}$ cells is unstable. Associated with the loss of resistance is a decrease in DHFR enzyme activity and a decrease in the mean number of dms per cell from about 64 under selective conditions to 30 after 1 day under non-selective conditions, 14 after 3 days and 6 after 14 days (C.J. Bostock, personal communication). If the amplified DNA sequences are carried on the dms, they too should decrease in amount under non-selective conditions. DNA preparations made from the samples

used for enzyme assays have been examined for the presence of the amplified DNA sequences visualized by ethidium bromide staining, and the number of DHFR genes in each has been estimated. Figs. 5.15. and 5.16. show the results. There is a noticable decrease in the intensity of the ethidium bromide - visualized bands, although the amount is difficult to assess quantitatively by eye. The important point is that these bands are still easily detectable after 14 days growth under non-selective conditions when there are only 10% of the dms remaining in the cells. Therefore not all the amplified DNA is associated with dms. The number of DHFR genes decreases somewhat during growth under non-selective conditions, but again about 40% of copies of the gene remain when when there are very few dms. Therefore not all of the amplified copies of the DHFR gene are associated with dms. However, the fact that there was some decrease in both of the characteristics measured suggests that some (very approximately a half) of the amplified DNA is carried on the dms. These results can be compared with the results obtained with the stably resistant EL4/11:MTX_R $1.1x10^{-3}M$ cells, where no significant change in the ethidium bromide fluorescence pattern was seen and there was no overall decrease in the number of DHFR genes (fig. 5.16.).

If only about a half of the amplified DNA in EL4/8:MTX_R $1.1x10^{-3}M$ cells is carried on dms, where is the other half? Two answers can be suggested: there could be

Fig. 5.15. Analysis of total cellular DNA from EL4/8:MTX$_R^{1.1x10^{-3}M}$ cells grown under non-selective conditions. DNA samples from cells on day 0, 3, 7, 10 or 14 of growth in the absence of MTX were digested with Eco RI and electrophoresed in a 0.75% agarose gel. The gel was stained with ethidium bromide, illuminated with u/v lamps, and the fluorescence was photographed. The first two tracks are marker DNA and EL4/WILD DNA digested with Eco RI.

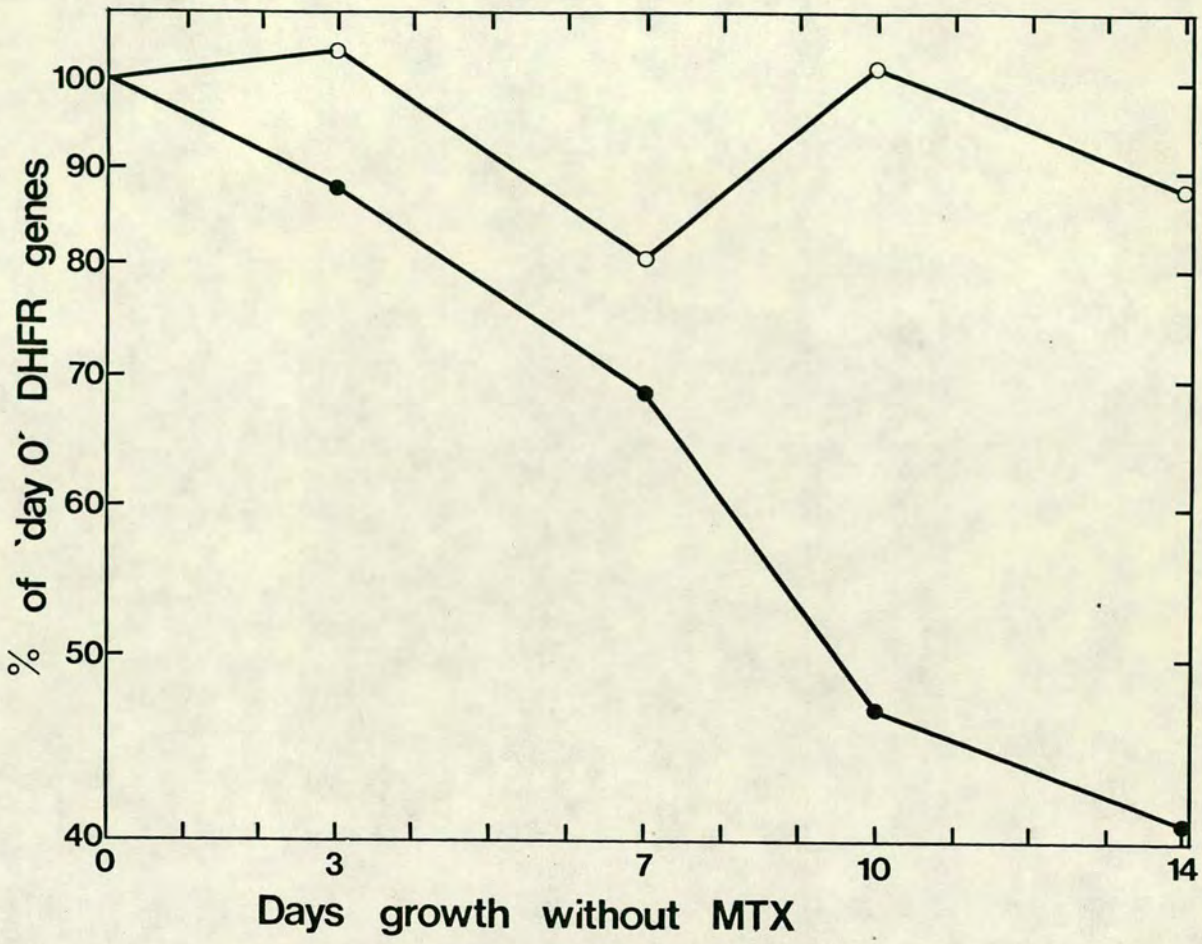


Fig. 5.16. Stability of DHFR gene number during growth in the absence of MTX. EL4/8:$MTX_R^{1.1x10^{-3}M}$ cells (—●—) or EL4/11:$MTX_R^{1.1x10^{-3}M}$ cells (—○—) were resuspended in medium lacking MTX and cells were harvested after 0, 3, 7, 10 and 14 days growth. Samples of total cellular DNA were prepared, digested with Eco RI, and electrophoresed in a 0.75% agarose gel. The DNA was transfered to nitrocellulose and the filter was hybridized with ^{32}P-labelled pDHFR 11. Bands of hybridization were located by autoradiography, cut out, and the radioactivity was measured in a scintillation counter. The amount of radioactivity from equal quantities of DNA, expressed as a % of the radioactivity in 'day 0' DNA of each cell line, is plotted here against the number of days growth without MTX.

smaller, submicroscopic, dm-like particles in these cells which are retained in a stable fashion when the cells are grown in non-selective medium, or the amplified DNA could be integrated into the visible chromosomes. These have been distinguished by separating chromosomes according to size on a sucrose gradient (carried out by C.J.Bostock) and analysing the fractions. If the amplified DNA is carried on very small particles and dms, it should be well separated from the bulk of the chromosomes and found at the top of the gradient; while if some amplified DNA is carried on the large chromosomes, it should be found all the way down the gradient. Fig 5.17. shows that both amplified DNA visualized. by ethidium bromide fluorescence and the DHFR gene are found all the way down the gradient. Microscopic examination of fractions showed that there was good separation between different size classes of chromosomes, although a few dms were seen in every fraction probably because they "stick" to other chromosomes. Some amplified DNA would be expected in all fractions because ring chromosomes and micronuclei of all sizes are present. However, the amount of DNA in these structures is far too small to account for the large amount of amplified DNA seen in all fractions. Mitochondrial DNA and cellular RNA served as internal markers: mitochondrial DNA was largely found in fraction 1 and RNA was concentrated in the topmost four or five fractions. Therefore the amplified DNA in the EL4/8:$MTX_R^{1.1x10^{-3}M}$ cells has a complex chromosomal location: about half of it is located on dms and about half is located on large chromosomes.

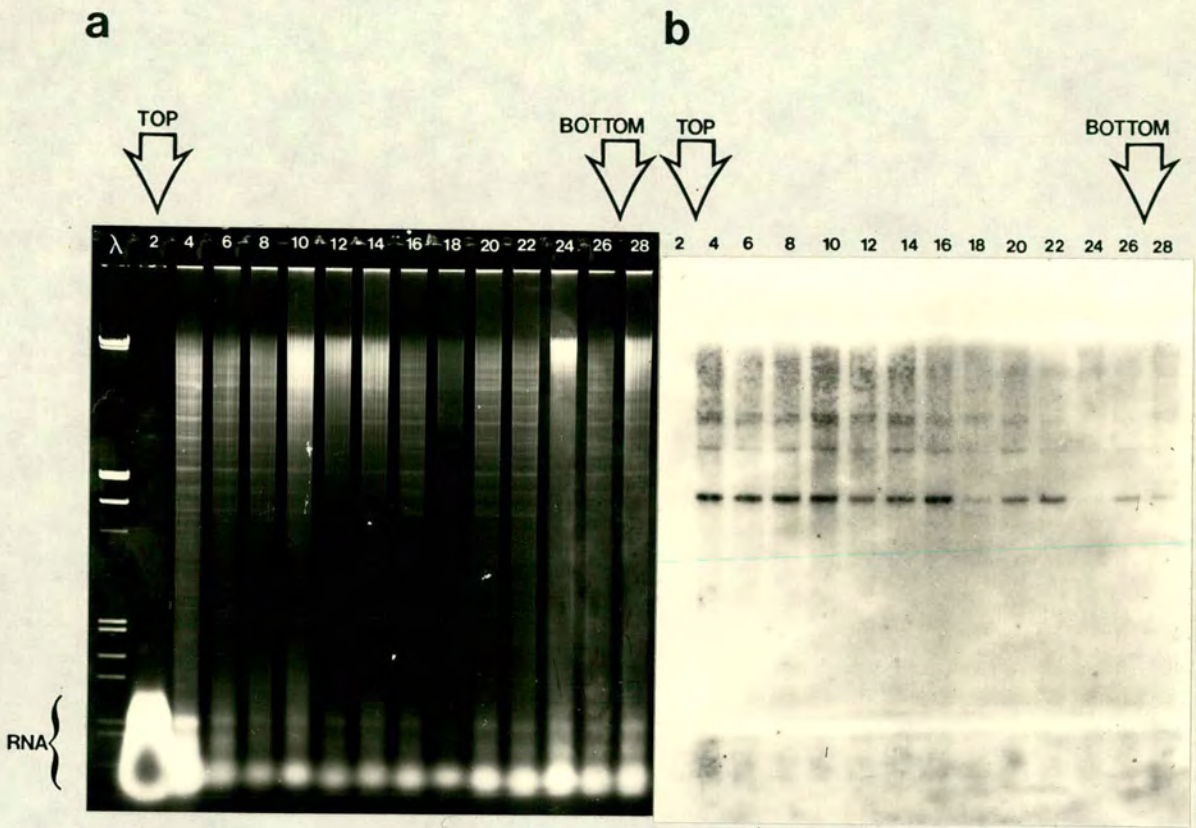


Fig. 5.17. Analysis of EL4/8:MTX$_R^{1.1x10^{-3}M}$ chromosome fractions after separation according to size on a sucrose density gradient. Chromosomes were fractionated on a sucrose gradient and nucleic acids were prepared from each fraction. The top of the gradient was fraction 1 and the bottom was fraction 32. Even-numbered fractions were digested with Eco RI and electrophoresed in a 0.75% agarose gel.

a. The gel was stained with ethidium bromide, illuminated with u/v lamps, and the fluorescence was photographed. A large amount of RNA can be seen in fractions 2 and 4.

b. DNA was transfered to nitrocellulose and the filter was hybridized with ^{32}P-labelled pDHFR 21 and autoradiographed.

CHAPTER 6

DISCUSSION PART II

6.1. GENE AMPLIFICATION IN PERSPECTIVE

Gene amplification is not a common mechanism by which mammals increase their capacity to synthesize a gene product (93). Perhaps the best counter-example is provided by the red blood cell which essentially contains only two polypeptides, α and β globin, but lacks a nucleus and consequently does not have even a single globin gene. Cells can produce large amounts of a protein by transcribing the gene at a high rate, making a stable and frequently translated mRNA molecule, and ensuring that the protein itself is stable. Organisms can synthesize large amounts of a product by selective prol-iferation of a specialized cell type. Why then, if all these normal mechanisms for synthesizing large amounts of a gene product exist, has gene amplification occurred in the cells characterized in this thesis? Firstly, since the cells being selecetd are growing in culture, in the absence of alterations of MTX transport or DHFR structure, every cell must produce large amounts of DHFR itself in order to survive. Thus potential for survival of the population by selective proliferation of a specialized DHFR-producing cell in a manner similar to the selective proliferation of specialized cells in a whole organizm is removed. Secondly, by defining the conditions as selective, that is as those that kill most cells, any normal mechanisms of increasing DHFR production will be insufficient since they will be possessed by most members of the population. Thirdly, it is the DHFR level compared to synthesis of

other proteins, RNA and DNA which must be increased. Thus, for example, a general increase in transcription rate or mRNA stability will be of no use. Fourthly, of all those mechanisms for increasing DHFR level which may be possible, only those which occur most frequently will actually be observed.

Those few systems where gene amplification has been found to occur in whole organisms are all cases where the final gene product is an RNA molecule and so the potential "amplification" steps inherent in protein synthesis cannot be used. This is particularly striking in the amphibians where very rapid ribosome assembly takes place during one stage of developmant. Many copies of the ribosomal RNA gene are amplified from the few carried in the germ-line DNA (section 6.2.2.), but no amplification of the ribosomal protein genes has been detected, although the rRNA and protein products are used in equimolar amounts.

6.2. ESTABLISHED EXAMPLES OF GENE AMPLIFICATION IN WHOLE ORGANISMS

Although, strictly, any change in the relative amounts of different parts of the genome could be called amplification, examples such as polysomy or simple chromosomal transloca--tions, and the polyteny that occurs in some insect chromosomes will be ommitted from this discussion and only examples where amplification is specific and of high order will be included. Two recent reviews covering this field have appeared (93, 176).

6.2.1. rDNA amplification in protozoa and slime molds

Several of the lower eukaryotes contain extra-chromosomal rDNA. The protozoon Tetrahymena pyriformis has, in the vegetative phase , a micronucleus and a macronucleus. The diploid micronucleus has a single integrated copy per haploid genome of the gene for the ribosomal RNA precursor (177); in contrast, the polyploid macronucleus lacks this integrated copy but has about 200 copies per haploid genome of extra-chromosomal rDNA which is found as paired linear palindromic molecules about 10 Kb long. Since the macronucleus is derived from the micronucleus after the sexual phase, amplification of the rDNA must occur. The process involves excision of the integrated copy, generation of a palindrome and amplification of the palindrome, but the details are unknown.

Extra-chromosomal rDNA has been found in the form of tandemly repeated units in linear and circular molecules in Paramecium tetraaurelia (178). It has also been found in Stylonochia (179, 180), but in this organism all the macronuclear DNA is found in the form of small "gene-sized" pieces. In slime molds, palindromic extra-chromosomal copies of rDNA have been found, ~60 Kb long in Physarum polycephalum and about 88 Kb long in Dictyostelium discoideum. It is thought that all these extra-chromosomal DNA molecules arise by amplification.

6.2.2. rDNA amplification during oogenesis.

rDNA amplification is a widespread but not universal feature of oogenesis. In the insects Dytiscus and Acheta, circular extrachromosomal copies of rDNA have been detected (181-183); in clams and in the worm Urechis a low level of amplification has been reported (184,185); among vertebrates high levels of amplification have been found in fish and amphibians (186,184,187,188), and a low level in humans (189). Reports of low levels of amplification must be interpreted with caution because of possible errors in the measurements.

The best-characterized system is Xenopus laevis (93). This frog carries about 500 slightly different copies of the rDNA repeat integrated in the germ line. In the primordial germ cell, a single one, or a few, of these repeats give rise to circular extrachromosomal copies which constitute about 20 to 50 times the haploid amount of rDNA. This level is maintained during proliferation of the germ cells in the growing tadpole, probably by autonomous replication of the amplified rDNA circles. As the tadpole enters metamorphosis and the germ cells start to undergo meiosis, then in spermatocytes the amount of rDNA falls to the haploid level, but in oocytes a second round of amplification takes place leading to about 2 500 times the haploid amount. While little is known about the mechanism of amplification in the primordial germ cells, it is well established that meiotic amplification in the oocyte takes place by a rolling circle mechanism.

6.3. DHFR GENE AMPLIFICATION

The evidence presented in this thesis suggests that DHFR gene amplification is a quite different process from the well-characterized examples of amplification in whole organisms discussed in section 6.2. The most important distinction is that DHFR gene amplification is an imprecise process, differing in the sequences involved and the amount of DNA in each cell line, and at least sometimes involving extensive sequence rearrangement (see below). Amplification by a rolling circle mechanism would generate identical copies of an intact portion of the genome; unequal crossing over between integral numbers of units of a repeating structure would also amplify a sequence intact, but if it occurred out-of-phase between non-integral numbers of repeat units it could generate sequence rearrangement. There are three reasons for thinking that unequal crossing over is not an important mechanism of amplification in these cells. Firstly, in those situations involving already tandemly repeated genes where it has been implicated on experimental or theoretical grounds (190-194), the size of the repeating unit and the distance of unequal crossover has been at least ten to one hundred times smaller than the amplified unit in MTX-resistant cells. Secondly, suitable arrays of units should be visible at the cytological level in cells resistant to high levels of MTX. dms are not candidates for such arrays; HSRs are more suitable. However, if unequal sister chromatid exchanges were sufficiently frequent to

double the number of DHFR genes in a high proportion of cells in a very few generations during selection for increased MTX resistance, they should also occur as frequently in cells growing at a fixed MTX concentration. Such unequal sister chromatid exchanges should then be visible cytologically as changes in the size of HSRs. While the size of the HSR has been correlated with the level of resistance (128), variation in size at a given level of resistance has not been noted. Thirdly, if unequal crossing over is the mechanism of gene amplification, and sequence rearrangements are introduced by out-of-phase unequal crossovers, such rearrangements should continue to be produced throughout selection: this is the "rearrangement switch-off" problem (section 5.2.). In order to explain why rearrangement ceases after the early stages in amplification, the ad hoc assumption would have to be made that alignment of repeat units is better in cells with multiple copies.

DHFR gene amplification itself seems to consist of two distinct processes. In the later stages of amplification there is a homogeneous population of amplified units, and changes in the number of DHFR genes, either increase during selection for a higher level of MTX resistance (section 5.2.1.) or decrease during growth under non-selective conditions(section 5.5.), take place in whole amplified units and do not involve change in the structure of the unit. In contrast, during the early stages of amplification, when the amplified unit is being generated, extensive DNA rearrangement takes place. In subsections 6.3.1

and 6.3.2. each of these processes is discussed.

6.3.1. Generation of the amplified unit

Two kinds of evidence show that DNA rearrangement has taken place during the generation of the amplified unit. Firstly, there is the direct evidence from the DHFR gene restriction patterns in the EL4 cells (section 3.10.) and possibly in S-180 cells (123). This evidence is direct in the sense that both the patterns before and after amplification are known, and the two are different. Secondly, there is indirect evidence from the restriction patterns of total amplified DNA in EL4 and PG19 cells: the patterns all contain unique fragments (section 5.2.). How can these be generated? Consider the simple case of amplifying intact a piece of chromosome. If only one amplified pattern was examined, then necessarily all the bands would be unique. If two were examined, then each would have some unique bands because overlapping but non-identical amplification units could have been chosen. However, if three patterns were examined, and no DNA rearrangement had taken place, then one of the three units must be the middle one and consequently can have no unique bands (see fig.6.1.). The observation that unique bands can be seen in three or more patterns shows that rearrangement must have taken place. Even if the two homologous chromosomes carrying the DHFR gene should differ in the neighbouring sequences, an extension of the above argument shows that only four unique patterns could be generated (fig.6.1.); five have been observed (figs 5.1. and

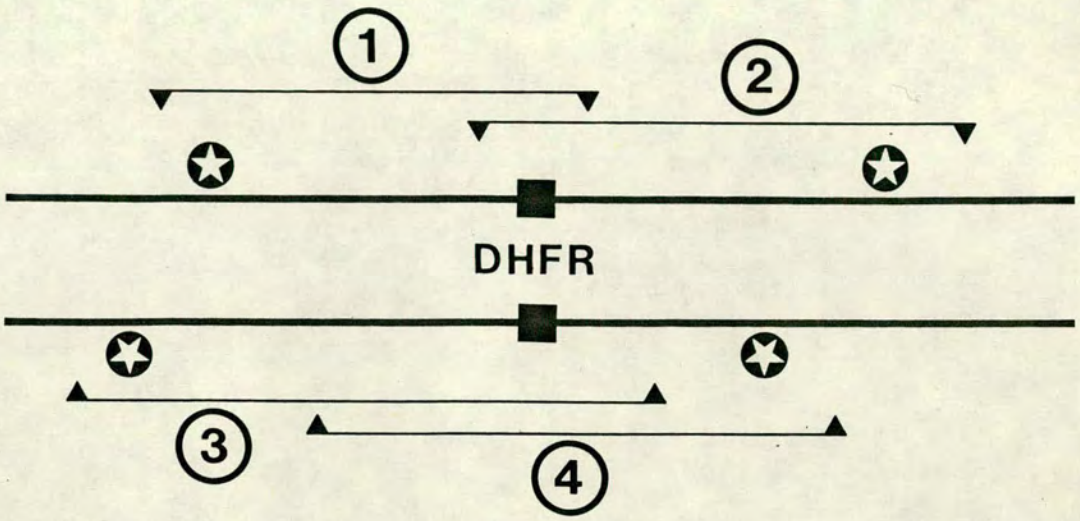


Fig. 6.1. Model showing how not more than four unique amplification units containing the DHFR gene could be derived from two homologous (but non-identical) chromosomes. Each of the amplification units numbered 1 - 4 could contain sequences which would give rise to unique bands (✪). Any fifth unit, however, could only contain sequences present in these four and therefore it could not give rise to unique bands.

Key: ▬■▬ chromosomal DNA containing DHFR gene.

▼——▼ amplification unit.

5.2.). The observation that extensive satellite DNA is amplified in the PG19T3:$MTX_R^{10^{-4}M}$ S1 cells suggests that DNA from this cell line contains a sixth set of sequences.

An understanding of how this DNA rearrangement has taken place will be essential to the understanding of how the amplified unit has been generated. It was suggested in section 4.4. that DNA rearrangement in the vicinity of the DHFR gene was more similar to the sequence non-specific DNA rearrangement which takes place in DNA-transformed cells than to the sequence-specific DNA rearrangement systems which have been identified in eukaryotes. This also seems to be true of the general DNA rearrangement in the amplified unit.

This suggests the possibility that the amplified unit really is generated by a spontaneous transformation event: that a cell can take up chromatin fragments from a neighbouring dying cell and express the DHFR gene. DNA-mediated transformation is carried out experimentally by forming a mixed precipitate of DNA and calcium phosphate (195) or DEAE dextran (196). It is not known precicely what the role of these carriers is: they may be important in protecting the DNA from nucleases in the serum contained in the cell culture medium or within the cell, or they may act on the cell membrane in such a way as to cause DNA uptake. In putative spontaneous transformation the first of these roles could be fulfilled by chromosomal proteins, and the second by the toxic effects of MTX leading to weakening or rupture of the cell membrane.

This hypothesis is testable: for example by examining MTX-resistant cells selected from a mixed culture of human and mouse cells for individuals with both types of DHFR gene. Such an experiment has not been done. However, it is perhaps significant that when mouse cells were transformed with DNA from hamster cells and selected for increasing levels of MTX resistance, all the four highly resistant clones examined contained amplified hamster DHFR gene and not amplified mouse DHFR genes (197). It seems that the incoming DNA was more easily formed into an amplifiable unit than the endogenous DNA. Even if transformation per se does not take place, that is, if the DNA which becomes the amplified unit comes from within the first resistant cell, possibly by some MTX-induced change, and not from the outside, the transformation experiments show that mouse cells are capable of ligating together and expressing many non-homologous DNA fragments. The explanation for the observed DNA rearrangement near the DHFR gene only in the MTX-resistant EL4 cells, could be that the DNA fragments contributing to the amplified units in these cells were smaller, possibly just because the endogenous nuclease levels in these cells are higher.

6.3.2. Increase in number of amplified units

Two major mechanisms seem to have been involved in the increase in the number of amplified units. Firstly, if the amplified DNA is carried on acentric chromatin fragments, as in some of the cells described in section 5.1. and unstably MTX-resistant S-180 cells (129), then it will not segregate using the mitotic apparatus at cell division, and may segregate unequally. It might be expected that, nevertheless, approximately equal numbers of dms would be found in each daughter cell; however, this simple view is probably wrong. A detailed study of mitosis in the dm-carrying MTX-resistant cells described here has not been carried out, but Levan and Levan (198) have made such a study in another mouse line, SEWARTC 13 clone 3R which has dms, although, so far as is known, these do not carry DHFR genes. In order to approximate as closely as possible to the natural conditions, all Levan and Levan's observations were made on cells which had not been treated with mitotic-arrest agents or given hypotonic swelling. They found that during prophase the dms were visible dispersed among the other chromosomes. When the nuclear membrane disintegrated, some of them spread into the cytoplasm. At metaphase, when the chromosomes were arranged in a ring in the equatorial plane with their centomeres pointing inwards, the dms were excluded from this assembly and were located at the periphery of the metaphase plate or in the cytoplasm. This was a charact--eristic peculiar to dms since small centric chromosomes were generally more centrally located than large ones,

and SEWARTC 13 clone 3R cells had a centric marker chromosome which was only slightly larger than the dms but was regularly found inside the ring. During metaphase, nucleolar material was pushed towards the periphery of the nucleus and therefore became associated with the dms. During anaphase, the chromosomes were pulled towards the poles; nucleolar material remaining associated with their ends was pulled with them and therefore a few of the dms also moved towards the poles. Many dms, however, remained separate from the main bodies of the chromosomes but associated with one another. At no stage in mitosis were sister dms specifically separated; generally they remained together. During interphase many dms appeared to form micro--nuclei; such micronuclei were seen in 14% of 500 cells examined.

Another study of the behaviour of dms during mitosis has been carried out by Barker and Hsu (199) using the human breast tumor line SW-613. They also observed that sister dms did not separate, but some of the pairs or small clumps of pairs became associated with other chromosomes and hence moved to daughter cell nuclei, while other pairs or groups seemed to be excluded from both daughter nuclei. These two papers show that dms from different sources behave in similar ways at mitosis.

The striking conclusion from these cytological observations is that essentially all the dms from the parental cell line can go to one of the daughter cells; thus in MTX-resistant lines, the number of DHFR genes carried on dms could almost be doubled in a single generation. This is

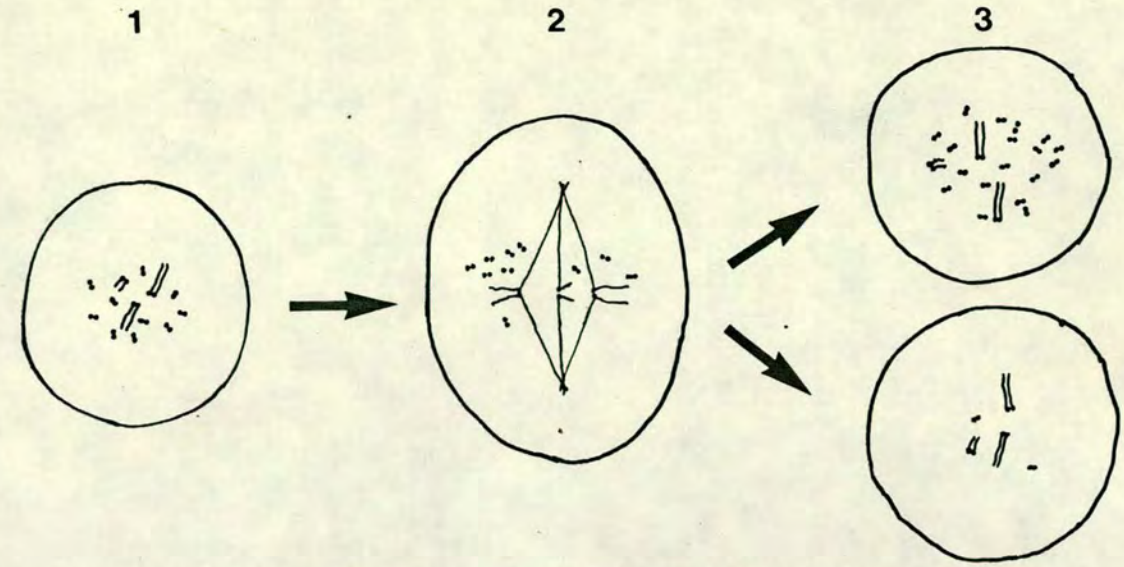
therefore potentially a very powerful mechanism of gene amplification. Evidence that such unequal segregation can occur in some of the cell lines described in this thesis is that in some metaphase spreads, almost all the dms can be seen lying associated with one another and separate from the other chromosomes (C.J.Bostock, personal communication).

The second major mechanism of increase in the number of amplified units depends on their having a circular structure. In such a case, any odd number of sister chromatid exchange events will generate a ring of twice the size. Such a ring can then go to only one of the daughter cells, increasing the number of DHFR genes in this cell, and thus its chance of survival, whereas the other daughter cell not receiving the ring will be selected against. This mechanism seems to have been important in the EL4 cells where acentric rings are very common, and rings of different sizes can be found within a single cell (section 5.1.). It may also have been important in the PG19 S1 cells where centric rings were observed at some stages (94). A ring with two functioning centomeres generated in this way will generally be disadvantageous to the cell since it will tend to be pulled towards both daughter cells at mitosis. However, under the highly selective conditions in these cultures, the few cells which avoided death would be selected. It has often been suggested that dms themselves are rings (200, 201), but this question cannot be answered by light microscopy and it remains a hypothesis. If true, it would suggest that this second mechanism of amplification could be involved in early as well as in late stages in

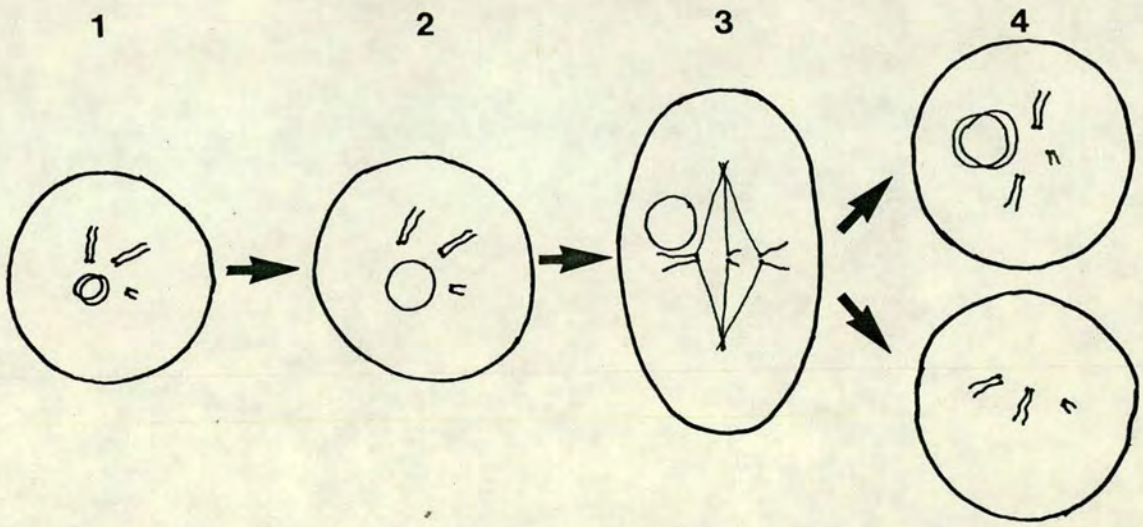
selection. These two mechanisms are illustrated in fig.6.2.

A third possible mechanism for increasing the number of amplified units is by replication more than once per cell generation. The fact that dms appear double at metaphase suggests that they generally replicate in phase with the other chromosomes, and "harlequin banding" studies confirm that this is true for most dms in the cells examined (202, 203). However, it is possible that DNA in micronuclei replicates out of phase with nuclear DNA. Evidence that such might be the case is tenuous: in some cells where the nuclear chromosomes have been examined at metaphase, the micronuclear chromosome has appeared to be at another stage in the replication cycle (204). There is no evidence that replication is more rapid rather than more slow in these cases, and only more rapid replication would lead to an increase in DNA amount; there is no evidence that this is a major mechanism of amplification.

A change occurring in some of the cells which is not a mechanism of amplification, but which is nevertheless relevant to the process, is stabilization. As indicated in section 5.2.3., a cell which carries its DHFR genes in a stable form has a selective advantage over a cell in which they are unstable, if cells are maintained in a constant MTX concentration. Stabilization can occur by acquisition of a functioning centomere. Centomeric acquisition is probably a rare chance event; its occurrence early in selection rather than late in selection could be



a. Unequal segregation of acentric chromatin fragments.
• = dms, ≈ = normal centric chromosomes.
1. cell after chromosome replication and before division containing 10 dms.
2. cell division: the dms do not associate with the mitotic apparatus and segregate unequally.
3. daughter cells (after chromosome replication): one has 18 dms, the other has 2.



b. Change in size of ring chromosome.
◎ = acentric ring chromosome, ≈ = normal centric chromosomes.
1. cell after chromosome replication and before division containing one ring chromosome.
2. a cross-over between the two sister ring chromatids produces a single ring chromatid of twice the original size.
3. at cell division the ring chromosome segregates with only one of the normal chromosome complements.
4. daughter cells (after chromosome replication): one has twice the amount of ring chromosome material in cell 1, the other has none.

Fig. 6.2. Proposed mechanisms of DNA amplification.

responsible for the observed chromosomal differences between the PG19 cells and the EL4 cells. HSRs on centric chromosomes have been found in MTX-resistant L5178Y cells (140) and MTX-resistant Chinese hamster cells (92, 128). Thus the very diverse chromosome morphologies seen in MTX-resistant cells can be explained as manifestations of a few underlying processes. This is summarised in fig.6.3.

The answer to the rearrangement switch-off problem (section 4.4.) is therefore that rearrangement generates an efficient amplicon: possibly a circular DNA molecule the size of one amplified unit. Once this has been formed, the more efficient amplification mechanisms described are the most frequent events leading to an increase in DHFR gene number, and consequently further DNA rearrangement is not observed.

6.3.3. Amplification in other MTX-resistant cells

The mechanisms discussed in sections 6.3.1 and 6.3.2. could thus account for amplification in the PG19 and EL4 cells. In the other cell lines examined, no comparable changes in chromosomes or total DNA were seen. Does this mean that the amplification process must be quite different in these cells? In the case of the L1210:MTX_R 10^{-4}M cells and the L5178Y:MTX_R 4.4×10^{-3}M cells, the relatively low degree of DHFR gene amplification and the difficulty in obtaining good quality DNA preparations from these cells could account for the absence of observed changes.

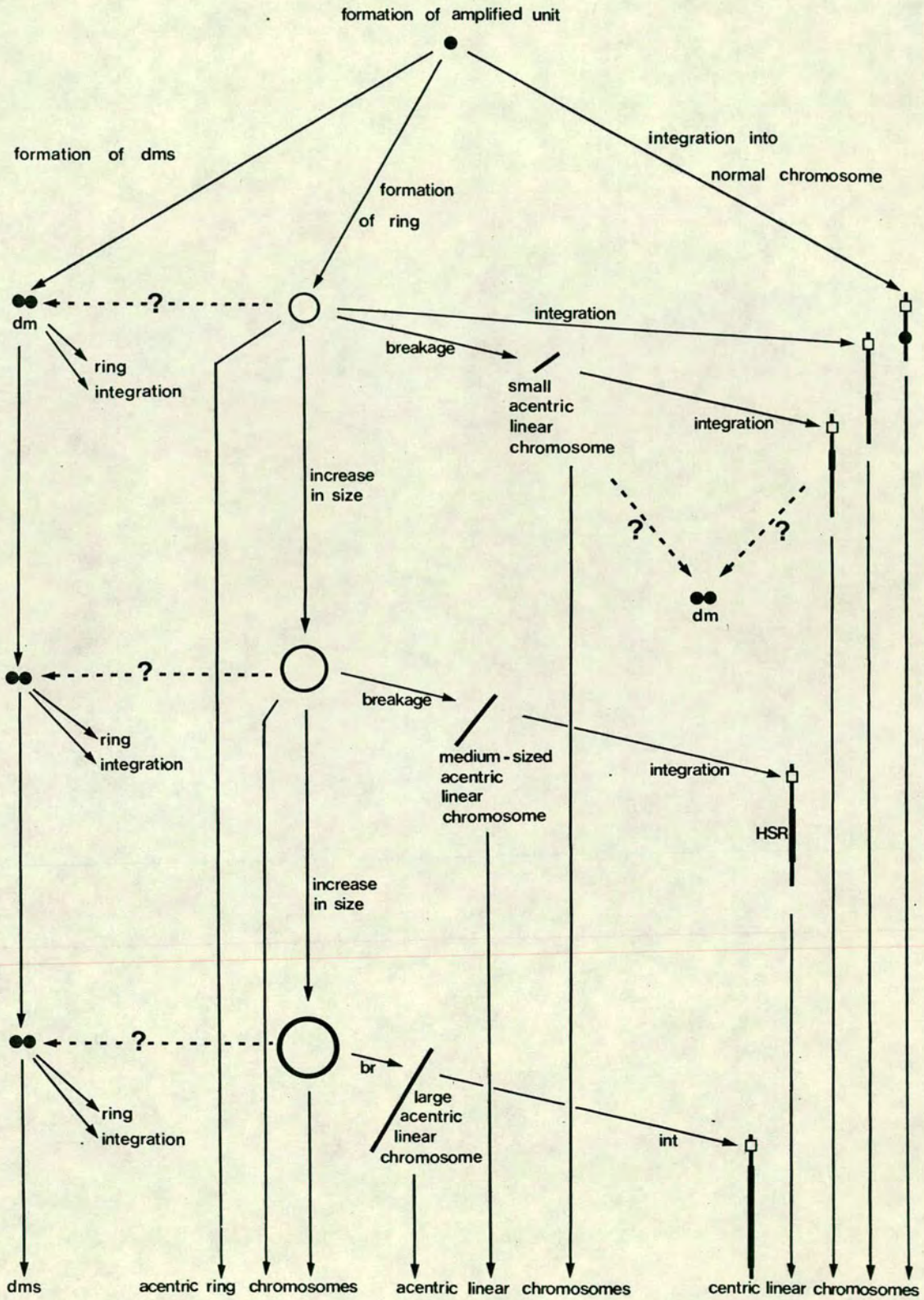


Fig. 6.3. Some possible inter-relationships between the chromosomal forms of dms, HSRs and rings seen in MTX-resistant cells.

●● = dm, ——— = HSR, —□— = centromere, ○ = ring.

In the case of LB1 trans:$MTX_R^{4.4x10^{-3}M}$ cells, such an explanation seems inadequate: the degree of amplification here is greater than in the PG19T3:$MTX_R^{10^{-4}M}$ S1 cells, and although good chromosome preparations were difficult to obtain, high molecular weight DNA could be isolated; yet bands of amplified DNA could not be seen (fig.5.2c.). This shows that the amplified unit does not contain sequences repeated within it which can give rise to visible bands in ethidium bromide stained gels. One explanation for this would be that it is small; another would be that amplified units within the population are heterogeneous. A small unit would also account for the lack of new chromosomal material visible in metaphase spreads; a heterogeneous collection of large amplified units of this number should be visible. Thus it is likely that the amplified unit in these cells is small; such a small unit could be generated and amplified in the ways outlined above.

6.4. DNA AMPLIFICATION IN SOMATIC CELLS

The processes thought to be involved in DHFR gene amplification in MTX-resistant cells are all potentially general ones: none depends specifically on DHFR or on MTX. Therefore it can be asked whether they are of more widespread occurrence. Ther are two situations where one might start to look. The first is where enzyme overproduction has been found as a mechanism of resistance to an inhibitor; the second is where dms or HSRs have been detected at the cytological level.

Inhibitor	Target enzyme	References
MTX	DHFR	see section 1.4.4. and chapter 3.
PALA	CAD	205,206,207,208.
6-azauridine, pyraazofurin	orotate phosphoribosyl transferase; orotidine 5'phosphate decarboxylase	209.
arabinosyl cytosine, hydroxyurea	ribonucleotide reductase	210,211,212,213.
5-fluoro-deoxyuridine	thymidylate synthetase	214,215,216.
cycloleucine	methionine adenosyl transferase	217,218.
β-aspartyl-hydroxamate	asparagine synthetase	219.
25-hydroxy-cholesterol	HMG-CoA reductase	220.
canavanine	argininosuccinate synthetase	221.

Table 6.1. Reports of enzyme overproduction associated with drug resistance in cultured mammalian cells.

Tumour	d	c	dm	ring	HSR	Comments	Ref
non-tumour	+	.	.	.	+	see text	223
HUMAN							
neuroblastoma	+	.	60/76	.	.		200
"	+	.	14/62	.	.		"
"	+	.	+	.	.		"
medulloblastoma	+	.	+	.	.		"
rhabdosarcoma	+	.	12/20	.	.		"
bronchial carcinoma	+	.	+	.	.		"
neuroblastoma	+	.	8/12	.	.		239
medulloblastoma	+	.	10/10	.	.	same patient	240
bone marrow	+	.	29/32	6/32	.	same patient	"
rhabdomyosarcoma R1	+	.	12/20	1/20	.		241
" R2	+	.	1/102	.	.	same patient	"
"	.	+	5-10%	.	.	same patient	"
thyroid carcinoma	.	+	+	+	.		242
astrocytoma	+	.	1/217	.	.		243
neuroblastoma	+	.	104/109	.	.		244
erythroleukemia	+	.	11/18	17/18	.		245
leukemia	+	.	7/25	.	.		226
"	+	.	25/25	2/25	.		"
"	+	.	8/25	.	.		"
"	+	.	11/25	14/25	.		"
"	+	.	14/25	.	.		"
"	+	.	9/25	9/25	.		"
"	+	.	17/25	.	.		"

Tumour	d	c	dm	ring	HSR	Comments	Ref
preleukemia	+	.	22/25	.	.		226
"	+	.	25/25	.	.		"
glioma 3	+	.	4(6.6%).		.	53 examined	224
" 4	+	.	9(9.7%).		.		"
" 10	+	.	6(11.8%).		.		"
" 18	+	.	2(5.4%).		.		"
" 22	+	.	89(80.2%).		.		"
" 26	+	.	21(61.8%).		.		"
" 29	+	.	70(100%) .		.		"
" 36	+	.	4(7.4%).		.		"
" 40	+	.	98(91.6%).		.		"
" 46	+	.	5(7.4%).		.		"
" 48	+	.	21(22.1%).		.		"
" 49	+	.	2(2.7%).		.		"
" 50	+	.	12(22.2%).		.		"
neuroblastoma	+	.	71/100	2/100	.		246
"	.	+	+	+	.		"
neuroblastoma	.	+	70%	.	.	42 days	247
"	.	+	14%	.	.	244 days	"
neuroblastoma	.	+	.	.	+		90
neuroblastoma	.	+	44/110	.	66/110		227
ovarian carcinoma	+	.	28/28	.	.	14 examined	225
"	+	.	7/28	.	.		"
breast	+	.	.	.	+		248
"	+	.	.	.	+		"
"	+	.	.	.	+		"

Tumour	d	c	dm	ring	HSR	Comments	Ref
oesophageal	+	.	.	.	+		248
pharangeal	+	.	.	.	+		"
colon: COLO 320	.	+	100%	5-10%	.	1½ months	228
" "	.	+	51%	.	94%	16 months	"
" COLO 321	.	+	few	.	98%		"
rectal carcinoma	.	+	+	.	.		"
breast,MDA-MB-143	.	+	5/50	.	.		203
" 157	.	+	2/50	.	.		"
" 175	.	+	5/50	.	.		"
" 231	.	+	21/50	.	.	see below	"
" 253	.	+	7/50	.	.		"
" 309	.	+	10/50	.	.		"
" 330	.	+	4/50	.	.		"
" 415	.	+	13/50	.	.		"
" 436	.	+	25/50	.	.		"
" BT-474	.	+	17/50	.	.		"
" SW-527	.	+	32/50	.	.	see below	"
" " 613	.	+	40/50	.	.		"
cervical, SW-732	.	+	40/50	.	.		"
breast,MDA-MB-231	.	+	.	.	+	see above	249
" SW-527	.	+	.	.	+	see above	"
breast tumour	+	.	.	.	+		"
breast, SW-1403	.	+	.	.	200/200		250

Tumour	d	c	dm	ring	HSR	Comments	Ref
MOUSE							
Rous sarcoma	+	.	+	.	.	9/50 tumours	251
SEWA and deriv.	+	+	+	+	few	multiple forms 252,253,254,202, 201,198.	
Ehrlich-Lettré	+	.	.	.	+		255
RAT							
H4-II-E-C3	.	+	.	+	+	see text	229
DOG							
venereal	+	.	.	.	+		235

Table 6.2. Published reports of dms, HSRs and associated ring chromosomes in cells of humans and animals which have not been selected for MTX resistance.

d = direct processing of tumour material for metaphase spreads

c = cultured cells examined

dm, ring, HSR } number of cells in which chromosome form is seen/total

Table 6.1. lists reports of enzyme overproduction in inhibitor-resistant cultured mammalian cells. In addition, esterase overproduction has been found in insecticide-resistant aphids of the species Myzus persicae (222). The molecular basis for overproduction has only been investigated in one system other than MTX resistance: PALA-resistant Chinese hamster cells. PALA (N-(phosphonacetyl)-L-aspartate) is an inhibitor of aspartate transcarbamylase, an enzyme essential for the de novo biosynthesis of UMP. Cells resistant to PALA overproduce CAD, the multifunctional protein containing the enzyme activities carbamyl-P synthetase, aspartate transcarbamylase and dihydroorotase. CAD overproduction is accompanied by CAD gene amplification (208). No cytological observations have been reported from these cells, and the size of the amplified unit and the involvement of other DNA sequences are unknown. From this data it appears that the process is similar to DHFR gene amplification. In none of the other systems has it been shown that a mechanism other that gene amplification is responsible for enzyme overproduction.

Table 6.2. lists reports of dms, HSRs and, where they are associated with these phenomena, ring chromosomes. This list is not exhaustive. It can be seen that, with one exception. these chromosomal forms are restricted to tumor cells. The exception is a short region on chromosome 14 of a normal woman (223) which was detected because it contained multiple nucleolus organizing regions (NORs). It could be simply the translocation of NORs from another chromosome to chromosome 14, or it could represent a low frequency of

HSRs in the population.

The true frequency of dms and HSRs in tumors is difficult to assess because these phenomena are easily overlooked unless they are specifically searched for, and dms can be confused with contaminants such as dirt or bacteria. The large number of reports of dms in neurogenic tumors in man almost certainly reflects a sampling bias: workers studying these tumors have been aware of the presence of dms and have looked for them. No systematic survey of the frequency of dms and HSRs has been carried out, and generally, workers who have reported their presence have not mentioned how many negative results they have obtained. Some limited figures are available. Mark (224) found dms in 13/53 (25%) of adult neurogenic tumors; Atkin and Pickthall (225) found them in 2/14 (14%) of cases of ovarian carcinoma; and Pierre et al (226) found them in 9/65 (14%) of cases of leukemia or preleukemia. Thus they appear to be widespread amongst different tumor types and may be present, perhaps, in one tenth to one third of all tumors.

It is significant that in some of these tumor lines, as in some of the MTX-resistant cell lines described in this thesis, dms and HSRs are associated. For example, Balaban-Manelbaum and Gilbert (227) found that in the human neuro-blastoma cell line CHP-126, 44/110 cells had dms and the other 66/110 cells in the same population had an HSR in the long arm of chromosome 5. All of these cells had two other marker chromosomes, suggesting a common origin for the two cell types. These workers therefore proposed that dms

were produced by fragmentation of the HSR: the converse of the view proposed here. Their observations are consistent with either view, and although in the EL4 cell population HSR-containing cells have arisen from dm-containing cells, possibly as outlined in fig.6.3., fragmentation of HSRs to produce dms cannot definitely be excluded as a mechanism of dm generation in other cell lines. However, a second report of HSRs arising from dms in a human carcinoma cell line (228) suggests that this was the more general occurrence. Cells from a colon adenocarcinoma were established in culture as COLO 320. In early cultures, almost all metaphases had from two to several hundred dms, and in addition 10% to 15% of metaphases had from one to seven ring chromosomes. After 16 months in culture, 49% of metaphases had 0 dms and 23% had one; 94% of metaphases, including all cells with less that eight dms, had a new marker chromosome possibly derived from the X chromosome and with an HSR on each arm.

Do these chromosomal changes reflect DNA amplification? In a trivial sense, any additional chromosomal material is DNA amplification. The important question is whether it is a high degree of amplification of a small amount of DNA, or one extra copy per cell of a large part of the genome. In other words, are the dms homogeneous? Do the HSRs have a repeating substructure? Cytological observations are not a good way of trying to answer these questions but, so far as they are capable, they do suggest that the answer is "yes". For example,

all observers have found that dms within a cell are uniform in staining properties, although not in size. If they were derived from a random selection of the genome, they would be expected to show differences. In the mouse, for instance, about 10% of the genome stains darkly after C banding (centromeric heterochromatin), yet 1 in 10 mouse dms is not C-band dark.

There is one instance where DNA amplification has been identified in HSRs: ribosomal DNA in the rat hepatoma line H-4-II-E-C3 (229). The origin of this cell line is shown in fig.6.4., so far as the details are known and published. The HSRs (called by the authors DSRs, for diferentially staining regions) were noticed after the cells had been in culture for several years. Cells had not been selected for properties other than the ability to grow fast in culture. Filter disk hybridization showed that there was about a ten-fold increase in the amount of rDNA, and <u>in situ</u> hybridization to metaphase chromosome spreads showed that about 90% of the total rDNA was located in the HSRs. Miller et al estimated that the HSRs contained about 3×10^5 Kb of DNA. The size of the rDNA repeat unit in the rat is unknown, but if the figure of 44Kb is taken from the mouse (234), and 200 copies per diploid genome are assumed (229), then 9 times the normal amount of rDNA is

$$9 \times 200 \times 44 = 7.9 \times 10^4 \text{ Kb}$$

which is only about a fifth of the total amount of HSR material. Thus the amplified unit is considerably larger than the rDNA repeat. Since ribosomal genes are clustered

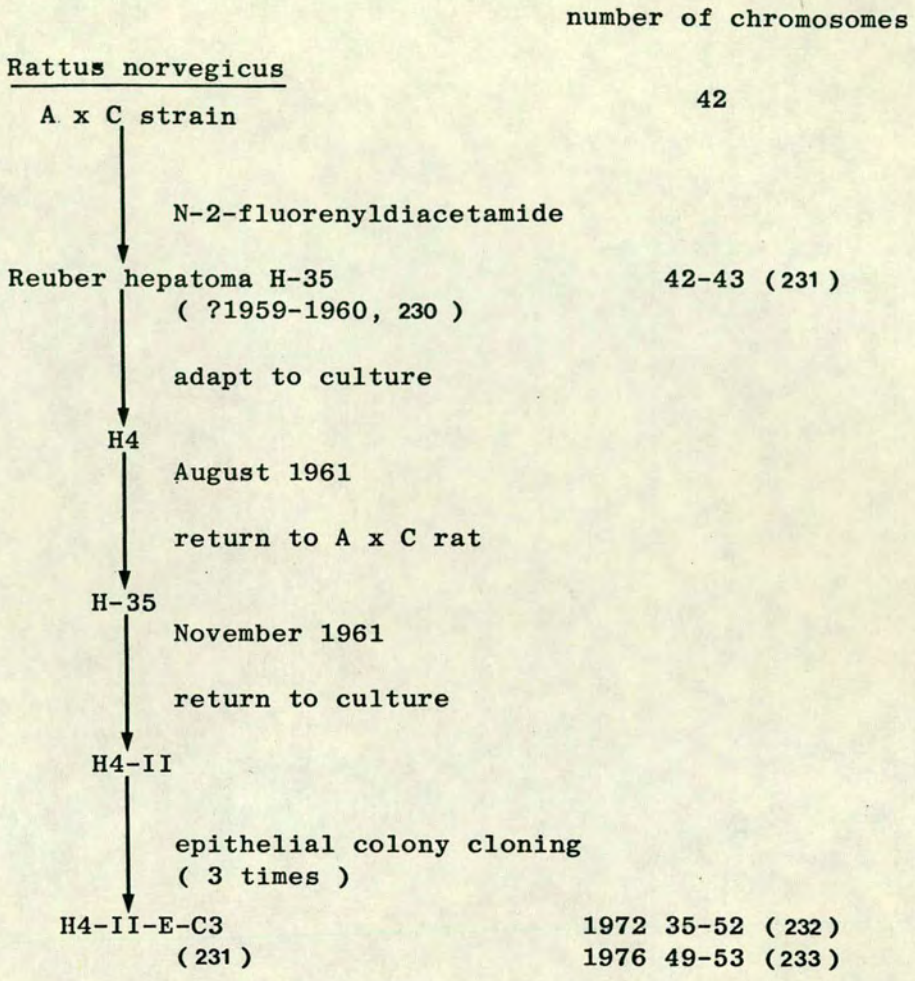


Fig. 6.4. Origin of the cell line H4-II-E-C3 containing chromosomes with amplified rDNA in HSR-like regions.

this suggests that either by chance the end of the cluster has been amplified, or that DNA rearrangement has taken place. The analogy with the DHFR gene amplification described in this thesis is therefore very striking. Another tumour, a venereal sarcoma in the dog, had an HSR which stained positively with the N-banding technique suggesting that it was a site of rRNA synthesis (235). and may represent another example of rDNA amplification. A cell line such as COLO 320 with an HSR apparently derived from a dm in a manner similar to that seen in EL4/8:$MTX_R^{1.1x10^{-3}M}$ cells (section 5.2.3.) must also contain amplified DNA. Thus it is likely that dms and HSRs do generally represent extensive amplification of a small part of the genome, and therefore that DNA amplification of this kind is a common feature of tumor cells.

What is its significance? Two kinds of answer can be given. It could be that it is DNA amplification which causes a normal cell to become a tumour cell; alternatively it could be that the ability to amplify DNA is a consequence of transformation: that a normal mechanism for the suppression of amplification has been lost. An experimental approach to this problem would be to ask whether non-transformed cells can amplify DHFR genes in culture and, if such amplification can occur, whether it is accompanied by transformation in the tumour-cell related sense. It should be noted that cells with dms can only remain in the tumour population for a large number of generations if they have a selective advantage over cells lacking dms, since dms are probably produced infrequently, replicated once per generation, and

often lost at cell division. It could be that in the tumor cell amplified DNA is mediating overproduction of a specific protein product in a manner directly analogous to the DHFR system, or its role could be more indirect: the question is open.

REFERENCES

(1) Office of population censuses and surveys.
Mortality statistics: cause.
Series DH2 no.5 (1978).
London. Her Majesty's Stationary Office.

(2) Scottish Health Statistics.
Report for 1978.
Edinburgh. Her Majesty's Stationary Office.

(3) Livingston RB and Carter SK.
Single agents in cancer chemotherapy.
IFI/Plenum. (1970).

(4) Werkheiser WC.
The biochemical, cellular and pharmacological action and effects of the folic acid antagonists.
Cancer Res 23 1277-1285 (1963).

(5) Hutchings BL, Stokstad ELR, Bohonos N and Slobodkin NH.
Isolation of a new Lactobacillus factor.
Science 99 371 (1944).

(6) Leuchtenberger R, Leuchtenberger C, Laszilo D and Lewisohn R.
The influence of "folic acid" on spontaneous breast cancer in mice.
Science 101 46 (1945).

(7) Lewisohn R, Leuchtenberger C, Leuchtenberger R and Keresztesy R.
The influence of liver L. casei factor on spontaneous breast cancer in mice.
Science 104 436-437 (1946).

(8) Farber S.
Some observations on the effect of folic acid antagonists on acute leukemia and other forms of incurable cancer.
Blood 4 160-167 (1949).

(9) Angier RB, Boothe JH, Hutchings BL, Mowat JH, Semd J, Stokstat ELR, Subbarow Y, Waller CW, Cosulich DB, Fahrenback MJ, Hultquist ME, Kuh E, Northey EL, Seeger DR, Sickels JP and Smith JM Jr.
The structure and synthesis of the liver L. casei factor.
Science 103 667-669 (1946).

(10) Seeger DR, Smith JM Jr and Hultquist ME.
Antagonist for pteroylglutamic acid.
J Am Chem Soc 69 2567 (1947).

(11) Farber S, Diamond LK, Mercer RD, Sylvester RF Jr and Wolff JA.
Temporary remissions in acute leukemia in children produced by the folic acid antagonist, 4-aminopteroyl-glutamic acid (aminopterin).
New England J Med 238 787-793 (1948).

(12) Meyer LM, Fink H, Sawitsky A, Bowen M and Ritz MD.
Aminopterin (a folic acid antagonist) in the treatment of leukemia.
Am J Clin Pathol 19 119-126 (1949).

(13) Berman L, Axelrod AR, Von der Heide EC and Sharp EA.
Use of a folic acid antagonist in chronic leukemia.
Am J Clin Pathol 19 127-133 (1949).

(14) Dameshek W.
The use of folic acid antagonists in the treatment of acute and subacute leukemia.
Blood 4 168-171 (1949).

(15) Goldin A, Venditti JM, Humphreys SR, Dennis D, Mantel N and Greenhouse SW.
A quantitative comparison of the antileukemic effectiveness of two folic acid antagonists in mice.
J Natl Cancer Inst 15 1657-1664 (1955).

(16) Cline MJ and Haskell CM.
Cancer chemotherapy.
Saunders (Philadelphia) 3rd edn (1979).

(17) Kosloski MJ, Rosen F, Milholland RJ and Papahadjopoulos D.
Effect of lipid vesicle (lipisome) encapsulation of methotrexate on its chemotherapeutic efficacy in solid rodent tumors.
Cancer Res 38 2848-2853 (1978).

(18) Chen ST, Kramer PA and Kildsig DO.
Interaction of methotrexate with polycationic carriers.
Life Sciences 23 365-374 (1978).

(19) Nichol CA and Welch AD.
On the mechanism of action of aminopterin.
Proc Soc Exp Biol Med 74 403-411 (1950).

(20) Futterman S.
Enzymatic reduction of folic acid and dihydrofolic acid to tetrahydrofolic acid.
J Biol Chem 228 1031-1038 (1957).

(21) Osborn MJ, Freeman M and Huennekens FM.
Inhibition of dihydrofolic reductase by aminopterin and amethopterin.
Proc Soc Exp Biol Med 97 429-431 (1958).

(22) Huennekens FM, Vitols KS, Whiteley JM and Neef VG.
Dihydrofolate reductase.
in Methods in cancer research, vol XIII, chapter V.
ed. Harris Busch.
Academic Press (new York, San Francisco, London) (1976)

(23) Greenberg DM, Tam B-D, Jenny E and Payes B.
Highly purified dihydrofolate reductase of calf thymus.
BBA 122 423-435 (1966).

(24) Bauman H and Wilson KJ.
Dihydrofolate reductase from bovine liver.
Enzymic and structural properties.
Eur J Biochem 60 9-15 (1975).

(25) Peterson DL, Gleisner JM and Blakley RL.
Bovine liver dihydrofolate reductase: purification and properties of the enzyme.
Biochemistry 14 5261-5267 (1975)

(26) Stone D and Phillips AW.
The amino acid sequence of dihydrofolate reductase from L1210 cells.
FEBS letters 74 85-87 (1977).

(27) Stone D, Paterson SJ, Raper JH and Phillips AW.
The amino acid sequence of dihydrofolate reductase from the mouse lymphoma L1210.
J Biol Chem 254 480-488 (1979).

(28) Smith SL, Patrick P, Stone D, Phillips AW and Burchall JJ.
Porcine liver dihydrofolate reductase (EC 1.5.1.3.): purification, properties and amino acid sequence.
J Biol Chem 254 11 475-11 484 (1979).

(29) Covey JM.
Minireview: Polyglutamate derivatives of folic acid coenzymes and methotrexate.
Life Sciences 26 665-678 (1980).

(30) Noronha JM and Aboobaker VS.
Studies on the folate compounds of human blood.
Arch Biochem Biophys 101 445-447 (1963).

(31) Whitehead VM.
Polygammaglutamyl metabolites of folic acid in human liver.
Lancet 1 743-745 (1973).

(32) Houlihan CM and Scott JM.
The identification of pteroylpentaglutamate as the major folate derivative in rat liver and the demonstration of its biosynthesis from exogenous ^{3}H pteroylglutamate.
BBRC 48 1675-1681 (1972).

(33) McGuire JJ, Coward JK, Hsieh P and Bertino JR.
Partial purification and characterization of folyl-polyglutamate.synthetase from rat liver.
Fed Proc 37 1347 (1978).

(34) Spronk AM.
Tetrahydrofolate polyglutamate synthesis in rat liver.
Fed Proc 32 471 (1973).

(35) Gawthorne JM and Smith RM.
The synthesis of pteroylpolyglutamates by sheep liver enzymes in vitro.
Biochem J 136 295-301 (1973).

(36) Coward JK, Parameswaran KN, Cashmore AR and Bertino JR.
7,8-Dihydropteroyl oligo-γ-L-glutamates: synthesis and kinetic studies with purified dihydrofolate reductase from mammalian sources.
Biochemistry 13 3899-3903 (1974).

(37) Dolnick BJ and Cheng Y.
Human thymidylate synthetase.
II. Derivatives of pteroylmono- and -polyglutamates as substrates and inhibitors.
J Biol Chem 253 3563-3567 (1978).

(38) Coward JK, Chello PL, Cashmore AR, Parameswaran KN, Deangelis LM and Bertino JR.
5-methyl 5678 tetrahydropteroyl oligo-γ-L-glutamates: synthesis and kinetic studies with methionine synthetase from bovine brain.
Biochemistry 14 1548-1552 (1975).

(39) Baggott JE and Krumdieck CL.
Folylpoly-γ-glutamates as cosubstrates of 10-formyl-tetrahydrofolate:5'-phosphoribosyl-5-amino-4-imidazole-carboxamide formyl transferase.
Biochemistry 18 1036-1041 (1979).

(40) McBurney MW and Whitmore GF.
Isolation and biochemical characterization of folate deficient mutants of Chinese hamster cells.
Cell 2 173-182 (1974).

(41) Taylor RT and Hanna ML.
Folate-dependent enzymes in cultured Chinese hamster cells: folylpolyglutamate synthetase and its absence in mutants auxotrophic for glycine + adenosine + thymidine.
Arch Biochem Biophys 181 331-334 (1977).

(42) Whitehead VM, Perrault MM and Stekner S.
Tissue-specific synthesis of methotrexate polyglutamates in the rat
Cancer Res 35 2985-2990 (1975).

(43) Jacobs SA, Derr CJ and Johns DG.
Accumulation of methotrexate diglutamate in human liver during methotrexate therapy.
Biochem Pharmacol 26 2310-2313 (1977).

(44) Rosenblatt DS, Whitehead VM, Vera N, Pottier A Dupont M and Vuchich M-J.
Prolonged inhibition of DNA synthesis associared with the accumulation of methotrexate polyglutamates by cultured human cells.
Mol Pharmacol 14 1143-1147 (1978).

(45) Whitehead VM and Rosenblatt DS.
Chemistry and biology of the pteridines.
Elsevier North-Holland (New York) (1979).

(46) Jacobs SA, Adamson RH, Chabner BA, Derr CF and Johns DG.
Stoichiometric inhibition of mammalian dihydrofolate reductase by the γ-glutamyl metabolite of methotrexate, 4-amino-4-deoxy-N^{10}-methylpteroylglutamyl-γ-glutamate.
BBRC 63 692-698 (1975).

(47) Donehower RC, Myers CE, Drake JC, Gallelli JF, Chabner BA and Engle R.
Comparison of radioimmunoassay (RIA) and competitive reductase binding assay (CRBA) for methotrexate (MTX).
Proc Am Assoc Cancer Res 19 172 (1978).

(48) Donehower RC, Hande KR, Drake JC and Chabner BA.
Presence of 2,4-diamino-N-10-methylpteroic acid after high-dose methotrexate.
Clin Pharmacol Ther 26 63-72 (1979).

(49) McCullough JL, Chabner BA and Bertino JR.
Purification and properties of carboxypeptidase G_1.
J Biol Chem 246 7201-7213 (1971).

(50) Donehower RC, Myers CE and Chabner BA.
Minireview: New developments on the mechanisms of action of antineoplastic drugs.
Life Sciences 25 1-14 (1979).

(51) Jacobs SA, Stroller RG, Chabner BA and Johns DG.
7-hydroxymethotrexate as a urinary metabolite in human subjects and rhesus monkeys receiving high dose methotrexate.
J Clin Invest 57 534-538 (1976).

(52) Johns DG and Loo TL.
Metabolite of 4-amino-4-deoxy-N^{10}-methylpteroylglutamic acid (methotrexate).
J Pharm Sci 56 356-359 (1967).

(53) Johns DG, Iannotti AT, Sartorelli AC, Booth BA and Bertino JR.
Tne identity of the rabbit-liver methotrexate oxidase.
BBA 105 380-382 (1965).

(54) Goldman ID.
The mechanism of action of methotrexate.
I. Interaction with a low-affinity intracellular site required for maximum inhibition of deoxyribonucleic acid synthesis in L-cell mouse fibroblasts.
Mol Pharmacol 10 257-274 (1974).

(55) White JC and Goldman ID.
Mechanism of action of methotrexate.
IV. Free intracellular methotrexate required to suppress dihydrofolate reduction to tetrahydrofolate by Ehrlich ascites tumor cells in vitro.
Mol Pharmacol 12 711-719 (1976).

(56) Borsa J and Whitmore GF.
Studies relating to the mode of action of methotrexate.
II. Studies on the sites of action in L-cells in vitro.
Mol Pharmacol 5 303-317 (1969).

(57) Borsa J and Whitmore GF.
Studies relating to the mode of action of methotrexate.
III. Inhibition of thymidylate synthetase in tissue culture cells and in cell-free systems.
Mol Pharmacol 5 318-332 (1969).

(58) Dolnick BJ and Cheng Y.
Human thymidylate synthetase derived from blast cells of patients with acute myelocytic leukemia.
J Biol Chem 252 7697-7703 (1977).

(59) Moran RG, Mulkins M and Heidelberger C.
Role of thymidylate synthetase activity in the development of methotrexate cytotoxicity.
PNAS 76 5924-5928 (1979).

(60) Schimke RT, Kaufman RJ, Alt FW and Kellems RF.
Gene amplification and drug resistance in cultured murine cells.
Science 202 1051-1055 (1978).

(61) Fischer GA.
Defective transport of amethopterin (methotrexate) as a mechanism of resistance to the antimetabolite in L5178Y leukemic cells.
Biochem Pharmacol 11 1233-1234 (1962).

(62) Kessel D, Hall TC, Roberts D and Wodinsky I.
Uptake as a determinant of methotrexate response in mouse leukemias.
Science 150 752-754 (1965).

(63) Sirotnak FM, Kurita S and Hutchinson DJ.
On the nature of a transport alteration determining resistance to amethopterin in the L1210 leukemia.
Cancer Res 28 75 (1968).

(64) Flintoff WF, Davidson VW and Siminovitch L.
Isolation and partial characterization of three methotrexate-resistant phenotypes from Chinese hamster ovary cells.
Somatic Cell Genet 2 245-261 (1976).

(65) Niethammer D and Jackson RC.
Changes of molecular properties associated with the development of resistance against methotrexate in human lymphoblastoid cells.
Europ J Cancer 11 845-854 (1975).

(66) Hill BT, Bailey BD, White JC and Goldman ID.
Characteristics of transport of 4-amino antifolates and folate compounds by two lines of L5178Y lymphoblasts, one with impaired transport of methotrexate.
Cancer Res 39 2440-2446 (1979).

(67) McCormick JI, Susten SS, Rader JI and Freishei M.
Studies of a methotrexate-binding fraction from L1210 lymphocyte plasma membranes.
Europ J Cancer 15 1377-1386 (1979).

(68) Biedler JL, Albrecht AM, Hutchison DS and Spengler BA.
Drug response, dihydrofolate reductase, and cytogenetics of amethopterin-resistant Chinese hamster cells in vitro.
Cancer Res 32 153-161).(1972).

(69) Albrecht A, Biedler JL and Hutchison DS.
Two different species of dihydrofolate reductase in mammalian cells differentially resistant to amethopterin and methasquin.
Cancer Res 32 1539-1546 (1972).

(70) Melera PW, Wolgemuth D, Biedler JL and Hession C.
Antifolate-resistant Chinese hamster cells. Evidence from independently derived sublines for the overproduction of two different dihydrofolate reductases encoded by different mRNAs.
J Biol Chem 255 319-322 (1980).

(71) Biedler JL, Albrecht AM and Spengler BA.
Biochemical and karyological properties of cells resistant to the quinazoline antifolate, methasquin.
Eur J Cancer 14 41-49 (1978).

(72) Jackson RC and Niethammer D.
Acquired methotrexate resistance in lymphoblasts resulting from altered kinetic properties of dihydrofolate reductase.
Europ J Cancer 13 567-575 (1977).

(73) Hakala MT, Zakrzewski SF and Nichol CA.
Relation of folic acid reductase to amethopterin resistance in cultured mammalian cells.
J Biol Chem 236 952-958 (1961).

(74) Fischer GA.
Increased levels of folic acid reductase as a mechanism of resistance to amethopterin in leukemic cells.
Biochem Pharmacol 7 75-77 (1961).

(75) Courtenay VD and Robbins AB.
Loss of resistance to methotrexate in L5178Y mouse leukemia grown in vitro.
J Natl Cancer Inst 49 45-53 (1972).

(76) Nakamura H and Littlefield JW.
Purification, properties and synthesis of dihydrofolate reductase from wild-type and methotrexate-resistant hamster cells.
J Biol Chem 247 179-189 (1972).

(77) Hanggi UJ and Littlefield JW.
Altered regulation of the rate of synthesis of dihydrofolate reductase in methotrexate-resistant hamster cells.
J Biol Chem 251 3075-3080 (1976).

(78) Alt FW, Kellems RE and Schimke RT.
Synthesis and degredation of folate reductase in sensitive and methotrexate-resistant lines of S-180 cells.
J Biol Chem 251 3063-3074 (1976).

(79) Chang SE and Littlefield JW.
Elevated dihydrofolate reductase messenger RNA levels in methotrexate-resistant BHK cells.
Cell 7 391-396 (1976).

(80) Kellems RE, Alt FW and Schimke RT.
Regulation of folate reductase synthesis in sensitive and methotrexate-resistant sarcoma 180 cells.
J Biol Chem 251 6987-6993 (1976).

(81) Bertino JR, Donohue DM, Gabrio BW, Silber R, Alenty A, Meyer M and Huennekens FM.
Increased levels of dihydrofolic reductase in leukocytes of patients treated with amethopterin.
Nature 193 140 (1962).

(82) Bertino JR, Donohue DM, Simmons B, Gabrio BW, Silber R and Huennekens FM.
The 'induction' of dihydrofolic reductase activity in leukocytes and erythrocytes of patients treated with amethopterin.
J Clin Invest 42 466-475 (1963).

(83) Roberts D and Hall TC.
Enzyme activities and deoxynucleoside utilization of leukemic leukocytes in relation to drug therapy and resistance.
Cancer Res 29 166-173 (1969).

(84) Kessel D, Hall TC and Roberts D.
Modes of uptake of methotrexate by normal and leukemic human leukocytes in vitro and their relation to drug response.
Cancer Res 28 564-570 (1968).

(85) Flintoff WF, Spindler SM and Siminovitch L.
Genetic characterization of methotrexate-resistant Chinese hamster ovary cells.
In Vitro 12 749-757 (1976).

(86) Littlefield JW.
Hybridization of hamster cells with high and low folate reductase activity.
PNAS 62 88-95 (1969).

(87) Alt FW, Kellems RE, Bertino JR and Schimke RT.
Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells.
J Biol Chem 253 1357-1370 (1978).

(88) Biedler JL, Albrecht AM and Hutchison DJ.
Cytogenetics of mouse leukemia L1210.
I. Association of a specific chromosome with dihydrofolate reductase activity in amethopterin-treated sublines.
Cancer Res 25 246-275 (1965).

(89) Benedict WF, Baker MS, Haroun L, Choi E and Ames BN.
Mutagenicity of cancer chemotherapeutic agents in the Salmonella/microsome test.
Cancer Res 37 2209-2213 (1977).

(90) Biedler JL and Spengler BA.
Metaphase chromosome anomaly: association with drug resistance and cell-specific products.
Science 191 185-187 (1976).

(91) Biedler JL and Spengler BA.
Quantitative relationship between a chromosome abnormality (HSR) and antifolate resistance associated with enzyme overproduction.
J Cell Biol 70 117a (1976).

(92) Nunberg JH, Kaufman RJ, Schimke RT, Urlab G and Chasin LA.
Amplified dihydrofolate reductase genes are localized to a homogeneously staining region of a single chromosome in a methotrexate-resistant CHO cell line.
PNAS 75 5553-5556 (1976).

(93) Bird AP.
Gene reiteration and gene amplification.
in Cell Biology: A Comprehensive Treatise, ed Goldstein L and Prescott DM. Volume 3, pp 61-111.
Academic Press (New York) (1980).

(94) Bostock CJ, Clark EM, Harding NGL, Mounts PM, Tyler-Smith C, van Heyningen V, and Walker PMB.
The development of resistance to methotrexate in a mouse melanoma cell line.
I. Characterization of the dihydrofolate reductases and chromosomes in sensitive and resistant cells.
Chromosoma 74 153-177 (1979).

(95) Jonasson J, Povey S and Harris H.
The analysis of malignancy by cell fusion.
VII. Cytogenetic analysis of hybrids between malignant and diploid cells and of tumours derived from them.
J Cell Sci 24 217-254 (1977).

(96) Gorer PA.
Studies in antibody response of mice to tumour innoculation.
British J Cancer 4 372-379 (1950).

(97) McKenzie IFC and Potter T.
Murine lymphocyte surface antigens.
Advances in Immunology 27 179-338 (1979).

(98) Law LW, Dunn TB, Boyle PJ and Miller JH.
Observations on the effect of a folic acid antagonist on transplantable lymphoid leukemias in mice.
J Natl Cancer Inst 10 179-192 (1949).

(99) Harding NGL, Martelli MF and Huennekens FM.
Amethopterin-induced changes in the multiple forms of dihydrofolate reductase from L1210 cells.
Arch Biochem Biophys 137 295-296 (1970).

(100) Fischer GA.
Nutritional and amethopterin-resistant characteristics of leukemic clones.
Cancer Res 19 372-376 (1959).

(101) Maio JJ & Schildkraut CL.
J Mol Biol 24 29-39 (1967)

(102) Gross-Bellard M, Oudet P and Chambon P.
Isolation of high-molecular-weight DNA from mammalian cells.
Eur J Biochem 36 32-38 (1973).

(103) Ross J.
A precursor of globin messenger RNA.
J Mol Biol 106 403-420 (1976).

(104) Chang ACY, Nunberg JH, Kaufman RJ, Erlich HA, Schimke RT and Cohen SN.
Phenotypic expression in E coli of a DNA sequence coding for mouse dihydrofolate reductase.
Nature 275 617-624 (1978).

(105) Zasloff M, Ginder GD and Felsenfeld G.
A new method for the purification and identification of covalently closed circular DNA molecules.
Nucleic Acids Res 5 1139-1152 (1978).

(106) Radloff R, Bauer W and Vinograd J.
A dye-buoyant-density method for the detection of closed circular duplex DNA: the closed circular DNA in HeLa cells.
PNAS 57 1514-1521 (1967).

(107) Maniatis T, Kee SG, Efstratiadis A and Kafatos FC.
Amplification and characterization of a β-globin gene synthesized in vitro.
Cell 8 163-182 (1976).

(108) Tiemeier DC, Tilgman SM and Leder P.
Purification and cloning of a mouse ribosomal gene fragment in coliphage lambda.
Gene 2 173-191 (1977).

(109) Southern EM.
Detection of specific sequences among DNA fragments separated by gel electrophoresis.
J Mol Biol 98 503-517 (1975).

(110) Maniatis T, Jeffrey A and Kleid DG.
Nucleotide sequence of the rightward operator of phage λ.
PNAS 72 1184-1188 (1975).

(111) Denhardt DT.
A membrane-filter technique for the detection of complementary DNA.
BBRC 23 641-652 (1966).

(112) Wahl GM, Stern M and Stark GR.
Efficient transfer of large DNA fragments from agarose gels to diazobenzoxymethyl-paper and rapid hybridization by using dextran sulphate.
PNAS 76 3683-3687 (1979).

(113) Velten J, Fukada K and Abelson J.
In vitro construction of bacteriophage λ and plasmid DNA molecules containing DNA fragments from bacteriophage T4.
Gene 1 93-106 (1976).

(114) Kafatos FC, Jones CW and Efstratiadis A.
Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure.
Nucleic Acids Res 7 1541-1552 (1979).

(115) Deeley RG, Gordon JI, Burns ATH, Mullinix KP, Binastein M and Goldberger RF.
Primary activation of the vitellogenin gene in the rooster.
J Biol Chem 252 8310-8319 (1977).

(116) Liu C-P, Slate DL, Gravel R and Ruddle FH.
Biological detection of specific mRNA molecules by microinjection.
PNAS 76 4503-4506 (1979).

(117) Bailey JM and Davidson N.
Methylmercury as a reversible denaturing agent for agarose gel electrophoresis.
Anal Biochem 70 75-85 (1976).

(118) Alwine JC, Kemp DJ and Stark GR.
Method for detection of specific RNAs in agarose gels by transfer to diazobenzoyloxymethyl-paper and hybridization with DNA probes.
PNAS 74 5350-5354 (1977).

(119) Gordon JI, Burns ATH, Christmann JL and Deeley RG.
Cloning of a double-stranded cDNA that codes for a portion of chicken preproalbumin.
J Biol Chem 253 8629-8639 (1978)

(120) Laemmli UK.
Cleavage of structural proteins during the assembly of the head of bacteriophage T4.
Nature 227 680-685 (1970).

(121) Higgins RC and Dahmus ME.
Rapid visualization of protein bands in preparative SDS-polyacrylamide gels.
Anal Biochem 93 257-260 (1979).

(122) Cleveland DW, Fischer SG, Kirschner MW and Laemmli UK.
Peptide mapping by limited proteolysis in SDS and analysis by gel electrophoresis.
J Biol Chem 242 1102-1106 (1977).

(123) Nunberg JH, Kaufman RJ, Chang ACY, Cohen SN and Schimke RT.
Structure and genomic organization of the mouse dihydrofolate reductase gene.
Cell 19 355-364 (1980).

(124) Abelson J.
A revolution in biology.
Science 209 1319-1321 (1980)
Quoting unpublished results of Krause G, Simonsen C and Schimke R.

(125) Waalwijk C and Flavell RA.
Msp I, an isoschizomer of Hpa II which cleaves both unmethylated and methylated Hpa II sites.
Nucleic Acids Res 5 3231-3236 (1978).

(126) Bird AP and Southern EM.
Use of restriction enzymes to study eukaryotic DNA methylation.
I. The methylation pattern in ribosomal DNA from Xenopus laevis.
J Mol Biol 118 27-47 (1978).

(127) van der Ploeg LHT, Groffen J and Flavell RA.
A novel type of secondary modification of two CCGG residues in the human γδβ-globin gene locus.
Nucleic Acids Res 8 4563-4574 (1980).

(128) Biedler JL, Melera PW and Spengler BA.
Specifically altered metaphase chromosomes in antifolate-resistant Chinese hamster cells that overproduce dihydrofolate reductase.
Cancer Genetics and Cytogenetics 2 47-60 (1980).

(129) Kaufman RJ, Brown PC and Schimke RT.
Amplified dihydrofolate reductase genes in unstably methotrexate-resistant cells are associated with double minute chromosomes.
PNAS 76 5669-5673 (1979).

(130) Waalwijk C and Flavell RA.
DNA methylation at a CCGG sequence in the large intron of the rabbit β-globin gene: tissue-specific variations.
Nucleic Acids Res 5 4631-4641 (1978).

(131) McGhee JD and Ginder GD.
Specific DNA methylation sites in the vicinity of the chicken β-globin genes.
Nature 280 419-420 (1979).

(132) van der Ploeg LTH and Flavell RA.
DNA methylation in the human γδβ-globin locus in erythroid and non-erythroid tissues.
Cell 19 947-958 (1980).

(133) Mandel JL and Chambon P.
DNA methylation: organ specific variations in the methylation pattern within and around ovalbumin and other chicken genes.
Nucleic Acids Res 7 2081-2103 (1979).

(134) Kuo MT, Mandel JL and Chambon P.
DNA methylation: correlation with DNase I sensitivity of chicken ovalbumin and conalbumin chromatin.
Nucleic Acids Res 7 2105-2113 (1979).

(135) Weintraub H and Groudine M.
Chromosomal subunits in active genes have an altered conformation.
Science 193 848-856 (1976).

(136) Wu C, Bingham PM, Livak KJ, Holmgren R and Elgin SRC.
The chromatin structure of specific genes:
I. Evidence for higher order domains of defined DNA sequence.
Cell 16 797-806 (1979)

(137) Wu C, Wong Y-C and Elgin SRC.
The chromatin structure of specific genes:.
II. Disruption of chromatin structure during gene activity.
Cell 16 807-814 (1979).

(138) Wu C.
The 5' ends of Drosophila heat shock genes in chromatin are hypersensitive to DNase I.
Nature 286 854-860 (1980).

(139) Stalder J, Larsen A, Engel JD, Dolan M, Groudine M and Weintraub H.
Tissue-specific DNA cleavages in the globin chromatin domain introduced by DNase I.
Cell 20 451-460 (1980).

(140) Dolnick BJ, Berenson RJ, Bertino JR, Kaufman RJ, Nunberg JH and Schimke RT.
Correlation of dihydrofolate reductase elevation with gene amplification in a homogeneously staining chromosomal region in L5178Y cells.
J Cell Biol 83 394-402 (1979).

(141) Bostock CJ and Clark EM.
Satellite DNA in large marker chromosomes of methotrexate-resistant mouse cells.
Cell 19 709-715 (1980).

(142) Sakano H, Huppi K, Heinrich G and Tonegawa S.
Sequences at the somatic recombination sites of immunoglobulin light-chain genes.
Nature 280 288-294 (1979).

(143) Early P, Huang H, Davis M, Calame K and Hood L.
An immunoglobulin heavy chain variable region gene is generated from three segments of DNA: V_H, D and J_H.
Cell 19 981-992 (1980).

(144) Alt FW, Enea V, Bothwell ALM and Baltimore D.
Activity of multiple light chain genes in murine myeloma cells producing a single, functional light chain.
Cell 20 1-12 (1980).

(145) Seidman JG and Leder P.
A mutant immunoglobulin light chain is formed by aberrant DNA- and RNA-splicing events.
Nature 286 779-783 (1980).

(146) Davis MM, Kim SK and Hood LE.
DNA sequences mediating class switching in α-immunoglobulins.
Science 209 1360-1365 (1980).

(147) Krawinkel U, Cramer M, Kindred B and Rajewsky K.
Isolated hapten-binding receptors of sensitized lymphocytes.
V. Cellular origin of receptor molecules.
Eur J Immunol 9 815-820 (1979).

(148) Kemp DJ, Harris AW, Cory S and Adams JM.
Expression of the immunoglobulin C_μ gene in mouse T and B lymphoid and myeloid cell lines
PNAS 77 2876-2880 (1980).

(149) Forster A, Hobart M, Hengartner H and Rabitts TH.
An immunoglobulin heavy chain gene is altered in two T-cell clones.
Nature 286 897-849 (1980).

(150) Williams RO, Young JR and Majiwa PAO.
Genomic rearrangements correlated with antigenic variation in trypanosoma brucei.
Nature 282 847-849 (1979).

(151) Hoeijmakers JHJ, Frasch ACC, Bernards A, Borst P and Cross GAM.
Novel expression-linked copies of the variant surface antigens in trypanosomes.
Nature 284 78-80 (1980).

(152) Turner M.
How trypanosomes change coats.
Nature 284 13-14 (1980).

(153) Nasmyth KA and Tatchell K.
The structure of transposable yeast mating type loci.
Cell 19 753-764 (1980).

(154) Hicks J, Strathern JN and Klar AJS.
Transposable mating type genes in Saccharomyces cerevisiae.
Nature 282 478-483 (1979).

(155) Finnegan DJ, Rubin GM, Young MW and Hogness DS.
Repeated gene families in Drosophila melanogaster.
CSHSQB 42 1053-1063 (1978).

(156) Strobel E, Dumsmuir P and Rubin GM.
Polymorphisms in the chromosomal locations of elements of the 412, copia and 297 dispersed repeated gene families in Drosophila.
Cell 17 429-439 (1979).

(157) Potter SS, Brorein WJ Jr, Dunsmuir P and Rubin GM.
Transposition of elements of the 412, copia and 297 dispersed repeated gene families in Drosophila.
Cell 17 415-427 (1979).

(158) Levis R, Dunsmuir P and Rubin GM.
Terminal repeats of the Drosophila transposable element copia: nucleotide sequence and genomic organization.
Cell 21 581-588 (1980).

(159) Dunsmuir P, Brorein WJ Jr, Simon MA and Rubin GM.
Insertion of the Drosophila transposable element copia generates a 5 base pair duplication.
Cell 21 575-579 (1980).

(160) Cameron JR, Loh EH and Davis RW.
Evidence for transposition of dispersed repetitive DNA families in yeast.
Cell 16 739-751 (1979).

(161) Farabaugh PJ and Fink GR.
Insertion of the eukaryotic transposable element Ty1 creates a 5 base pair duplication.
Nature 286 352-356 (1980).

(162) Gafner J and Philippsen P.
The yeast transposon Ty1 generates duplications of target DNA on insertion.
Nature 286 414-418 (1980).

(163) Maitland NJ and McDougall JK.
Biochemical transformation of mouse cells by fragments of Herpes Simplex Virus DNA.
Cell 11 233-241 (1977).

(164) Wigler M, Silverstein S, Lee L-S, Pellicer A, Cheng Y and Axel R.
Transfer of purified Herpes Simplex Virus thymidine kinase gene to cultured mouse cells.
Cell 11 223-232 (1977).

(165) Pellicer A, Wigler M, Axel R and Silverstein S.
The transfer and stable integration of the HSV thymidine kinase gene into mouse cells.
Cell 14 133-141 (1978).

(166) Wigler M, Sweet R, Sim GK, Wold B, Pellicer A, Lacy E, Maniatis T, Silverstein S and Axel R.
Transformation of mammalian cells with genes from prokaryotes and eukaryotes.
Cell 16 777-785 (1979).

(167) Scagnos GA, Huttner KM, Silverstein S and Ruddle FH.
Molecular analysis of chromosome-mediated gene transfer.
PNAS 76 3987-3990 (1979).

(168) Pellicer A, Robins D, Wold B, Sweet R, Jackson J, Lowy I, Roberts JM, Sim GK, Silverstein S and Axel R.
Altering genotype and phenotype by DNA-mediated gene transfer.
Science 209 1414-1422 (1980).

(169) Temin HM.
Origin of retroviruses from cellular moveable genetic elements.
Cell 21 599-600 (1980).

(170) Joho R and Weissman IL.
V-J joining of immunoglobulin κ genes occurrs on one homologous chromosome.
Nature 284 179-181 (1980).

(171) Perry RP, Kelley DE, Coleclough C, Steidman JG, Leder P, Tonegawa S, Matthyssens G and Weigert M.
Transcription of mouse κ chain genes: implications for allelic exclusion.
PNAS 77 1937-1941 (1980).

(172) Cheng SM and Schildkraut CL.
A family of moderately repetitive sequences in mouse DNA.
Nucleic Acids Res 8 4075-4090 (1980).

(173) Heller R and Arnheim N.
Structure and organization of the highly repeated and interspersed 1.3kb Eco RI-Bgl II sequence family in mice.
Nucleic Acids Res 8 5031-5042 (1980).

(174) Kit S.
Equilibrium sedimentation in density gradients of DNA preparations from animal tissues.
J Mol Biol 3 711-716 (1961).

(175) Jahn CL, Hutchison CA III, Phillips SJ, Weaver S, Haigwood NL, Voliola CF and Edgell MH.
DNA sequence organization of the β-globin complex in the Balb/c mouse.
Cell 21 159-168 (1980)

(176) Long EO and Dawid IB.
Repeated genes in eukaryotes.
Ann Rev Biochem 49 727-764 (1980).

(177) Yao M-C and Gall JG.
A single integrated gene for ribosomal RNA in a eukaryote Tetrahymena pyriformis.
Cell 12 121-132 (1977).

(178) Findly RC and Gall JG.
Free ribosomal RNA genes in Paramecium are tandemly repeated.
PNAS 75 3312-3316 (1978).

(179) Prescott DM, Murti KG and Bostock CJ.
Genetic apparatus of Stylonichia sp.
Nature 242 576-600 (1973).

(180) Lipps HJ and Steinbruck G.
Free genes for rRNAs in the macronuclear genome of the ciliate Stylonichia mytilus.
Chromosoma 69 21-26 (1978).

(181) Gall JG and Rochaix J-D.
The amplified ribosimal DNA of Dytiscid beetles.
PNAS 71 1819-1823 (1974).

(182) Trendenburg MF, Scheer U, Zentgraf H and Franke WW.
Heterogeneity of spacer lengths in circles of amplified ribosomal DNA of two insect species, Dytiscus marginalis and Acheta domesticus.
J Mol Biol 108 453-470 (1976).

(183) Cave MD.
Length heterogeneity of amplified circular rDNA molecules in oocytes of the house cricket Acheta domesticus (Orthoptera: Gryllidae).
Chromosoma 71 15-27 (1979).

(184) Brown DD and Dawid IB.
Specific gene amplification in oocytes.
Science 160 272-280 (1968).

(185) Dawid IB and Brown DD.
The mitochondrial and ribosomal DNA components of oocytes of Urechis caupo.
Dev Biol 22 1-14 (1970).

(186) Vincent WS, Halvorsen HO, Chen H-R and Shin D.
A comparison of ribosomal gene amplification in uni- and multi-nucleolate oocytes.
Exp Cell Res 57 240-250 (1969).

(187) Gall JG.
Differential synthesis of the genes for ribosomal RNA during amphibian oogenesis.
PNAS 60 553-560 (1969).

(188) Macgregor HC.
Nucleolar DNA in oocytes of Xenopus laevis.
J Cell Sci 3 437-444 (1968).

(189) Wolgemuth DJ, Jagiello GM and Henderson AS.
Quantitation of ribosomal RNA genes in fetal human oocyte nuclei using rRNA:DNA hybridization in situ.
Exp Cell Res 118 181-190 (1979).

(190) Szostak JW and Wu R.
Unequal crossing over in the ribosomal DNA of Saccharomyces cerevisiae.
Nature 284 426-430 (1980).

(191) Petes TD.
Unequal meiotic recombination within tandem arrays of yeast ribosomal DNA genes.
Cell 19 765-774 (1980).

(192) Tartof KD.
Unequal mitotic sister chromatid exchange and disproportionate replication as mechanisms regulating ribosomal RNA gene redundancy.
CSHSQB 38 491-500 (1973).

(193) Smith GP.
Unequal crossover and the evolution of multigene families
CSHSQB 38 507-513 (1973).

(194) Goossens M, Dozy AM, Embury SH, Zachariades Z, Hadjiminas MG, Stramatoyannopoulos G and Kan YW.
Triplicated α-globin loci in humans.
PNAS 77 518-521 (1980).

(195) Graham FL and van der Eb AJ.
A new technique for the assay of infectivity of human adenovirus 5 DNA.
Virology 52 456-467 (1973).

(196) McCutchan JH and Pagano JS.
Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl dextran.
J Natl Cancer Inst 41 351-357 (1968).

(197) Wigler M, Perucho M, Kurtz D, Dana S, Pellicer A, Axel R and Silverstein S.
Transformation of mammalian cells with an amplifiable dominant-acting gene.
PNAS 77 3567-3570 (1980).

(198) Levan A and Levan G.
Have double minutes functioning centromeres?
Hereditas 88 81-92 (1978).

(199) Barker PE and Hsu TC.
Are double minutes chromosomes?
Exp Cell Res 113 456-458 (1978).

(200) Cox D, Yuncken C and Spriggs AI.
Minute chromatin bodies in malignant tumors of childhood.
Lancet II 55-58 (1965).

(201) Levan A, Levan G and Mandahl N.
A new chromosome type replacing the double minutes in a mouse tumor.
Cytogenet Cell Genet 20 12-23 (1978).

(202) Levan A, Levan G and Mitelman F.
Chromosomes and cancer.
Hereditas 86 15-30 (1977).

(203) Barker PE and Hsu TC.
Double minutes in human carcinoma cell lines, with special reference to breast tumors.
J Natl Cancer Inst 62 257-262 (1979).

(204) Ledbetter DH, Riccardi VM, Au WW, Wilson DP and Holmquist GP.
Ring chromosome 15: phenotype, Ag-NOR analysis, secondary aneuploidy, and associated chromosome instability.
Cytogenet Cell Genet 27 111-122 (1980)

(205) Kempe TD, Swyryd EA, Bruist M and Stark GR.
Stable mutants of mammalian cells that overprobuce the first three enzymes of pyrimidine nucleotide biosynthesis.
Cell 9 541-550 (1976).

(206) Coleman PF, Suttle DP and Stark GR.
Purification from hamster cells of the multifunctional protein that initiates de novo synthesis of pyrimidine nucleotides.
J Biol Chem 252 6379-6385 (1977).

(207) Padgett RA, Wahl GM, Coleman PF and Stark GR.
N-(Phosphonacetyl)-L-aspartate-resistant hamster cells overaccumulate a single mRNA coding for the multi-functional protein that catalyses the first three steps of UMP synthesis.
J Biol Chem 254 974-980 (1979).

(208) Wahl GM, Padgett RA and Stark GR.
Gene amplification causes overproduction of the first three enzymes of UMP synthesis in N-(Phosphonacetyl)-L-aspartate-resistant hamster cells.
J Biol Chem 254 8679-8689 (1979).

(209) Suttle DP and Stark GR.
Coordinate overproduction of orotate phosphoribosyl transferase and orotidine-5'-phosphate decarboxylase in hamster cells resistant to pyrazofurin and 6-azauridine.
J Biol Chem 254 4602-4607 (1979).

(210) Meuth M and Green H.
Alterations leading to increased ribonucleotide reductase in cells selected for resistance to deoxynucleosides.
Cell 3 367-374 (1974).

(211) Lewis WH, Kuzik BA and Wright JA.
Assay of ribonucleotide reduction in nucleotide-permeable hamster cells.
J Cell physiol 94 287-298 (1978).

(212) Lewis WH and Wright JA.
Isolation of hydroxy-urea resistant CHO cells with altered levels of ribonucleotide reductase.
Somatic Cell Genet 5 83 (1979).

(213) Kuzik BA and Wright JA.
Hydroxyurea-resistant mouse L cells with elevated levels of drug-resistant ribonucleotide reductase activity.
Biochem Genet 18 311-332 (1980).

(214) Baskin F, Carlin SC, Kraus P, Friedkin M and Rosenberg RN.
Experimental chemotherapy of neuroblastoma.
II. Increased thymidylate synthetase activity in a 5-fluorodeoxyuridine-resistant variant of mouse neuroblastoma.
Mol Pharmacol 11 105-117 (1975).

(215) Wilkinson DS, Solomonson LP and Cory JG.
Increased thymidylate synthetase activity in a 5-fluorodeoxyuridine-resistant Novikoff hepatoma cells.
Proc Soc Exp Biol Med 154 368-371 (1977).

(216) Priest DG, Ledford BE and Doig MT.
Increased thymidylate synthetase in 5-fluorodeoxyuridine-resistant cultured hepatoma cells.
Biochem Pharmacol 29 1549-1554 (1980).

(217) Caboche M.
Methionine metabolism in BHK cells: the regulation of methionine adenosyl transferase.
J Cell Physiol 92 407-427 (1977).

(218) Caboche M and Mulsant P.
Selection and prelininary characterization of cycloleucine-resistant CHO cells affected in methionine metabolism.
Somatic Cell Genet 4 407-421 (1978).

(219) Gantt JS, Chiang CS, Hatfield GW and Arfin SM.
Chinese hamster ovary cells resistant to β-aspartyl-hydroxamate contain increased levels of asparagine synthetase.
J Biol Chem 255 4808 -4813 (1980).

(220) Sinensky M.
Isolation of a mammalian cell mutant resistant to 25-hydroxy cholesterol.
BBRC 78 863-867 (1977).

(221) Kimball ME and Jacoby LB.
Purification and properties of argininosuccinate synthetase from normal and canavanine-resistant human lymphoblasts.
Biochemistry 19 705-709 (1980).

(222) Devonshire AL and Sawicki RM.
Insecticide-resistant Myzus persicae as an example of evolution by gene duplication.
Nature 280 140-141 (1979).

(223) Lau YF, Wertelecki W, Pfeiffer RA and Arrighi FE.
Cytological analysis of a 14p+ variant by means of N-banding and combination of silver staining and chromosome banding.
Hum Genet 46 75-82 (1979).

(224) Mark J.
Chromosomal characteristics of neurogenic tumours in abults.
Hereditas 68 61-100 (1971).

(225) Atkin NB and Pickthall VJ.
Chromosomes 1 and 14 in ovarian cancers.
Heterochromatic variants and structural changes.
Hum Genet 38 25-33 (1977).

(226) Pierre RV, Hoagland KC and Linman JW.
Minichromosomes in human preleukemia and leukemia.
Cancer 27 160-175 (1971).

(227) Balaban-Malenbaum G and Gilbert F.
Double minute chromosomes and the homogeneously staining regions of a human neuroblastoma cell line.
Science 198 739-741 (1977).

(228) Quinn LA, Moore GE, Morgan RT and Woods LK.
Cell lines from human colon carcinoma with unique cell products, double minutes and homogeneously staining regions.
Cancer Res 39 4914-4929 (1979).

(229) Miller OJ, Tantravahi R, Miller DA, Yu LC, Szabo P and Prensky W.
Marked increase in ribosomal gene multiplicity in a rat hepatoma cell line.
Chromosoma 71 183-195 (1979).

(230) Reuber MD.
A transplantable bile-secteting hepatocellular carcinoma in the rat.
J Natl Cancer Inst 26 891-897 (1961).

(231) Pitot HC, Peraino C, Morse PA Jr and Potter VR.
Hepatomas in tissue culture compared with adapting liver in vitro.
Natl Cancer Inst Monograph 13 229-242 (1964).

(232) Miller DA, Dev VG, Borek C and Miller OJ.
The quinacrine fluorescent and Giemsa banded karyotype of the rat, Rattus norwegicus, and banded chromosome analysis of transformed and malignant rat liver cell lines.
Cancer Res 32 2375-2382 (1972).

(233) Mullen VT and Barnett CA.
Banded karyotypes of H-4-IIE-C3 rat hepatoma cells grown in vitro.
In Vitro 12 658-664 (1976).

(234) Corey S and Adams JM.
A very large repeating unit of mouse DNA containing the 18S, 28S and 5.8S rRNA genes.
Cell 11 795-805 (1977).

(235) Oshimura M, Sasaki M and Makino S.
Chromosomal banding patterns in primary and transplanted venereal tumors of the dog.
J Natl Cancer Inst 51 1197-1203 (1973).

(236) Schimke RT, Kaufman RJ, Nunberg JH and Dana SL.
Studies on the amplification of dihydrofolate reductase genes in methotrexate-resistant cultured mouse cells.
CSHSQB XLIII 1297-1303 (1978).

(237) Kellems RE, Morhenn VB, Pfendt EA, Alt FW and Schimke RT.
Polyoma virus and cyclic AMP-mediated control of dihydrofolate reductase mRNA abundance in methotrexate-resistant mouse fibroblasts.
J Biol Chem 254 309-318 (1979).

(238) Melera PW, Lewis JA, Biedler JL and Hession C.
Antifolate-resistant Chinese hamster cells.
Evidence for dihydrofolate reductase gene amplification among indepently derived sublines overproducing different dihydrofolate reductases.
J Biol Chem 255 7024-7028 (1980).

(239) Lubs HA Jr and Salmon JH.
The chromosomal complement of human solid tumors.
II. Karyotypes of glial tumors.
J Neurosurg 22 160-168 (1965).

(240) Lubs HA Jr, Salmon JH and Flanigen S.
Studies of a glial tumor with multiple minute chromosomes.
Cancer 19 591-599 (1966).

(241) White L and Cox D.
Chromosome changes in a rhabdomyosarcoma during recurrence and in cell culture.
Br J Cancer 21 684-696 (1977).

(242) Jones GW, Simkovik D, Biedler JL and Southan CM.
Human anaplastic thyroid carcinoma in tissue culture.
Proc Soc Exp Biol Med 126 426-428 (1967).

(243) Hansteen IL.
Chromosome studies in glial tumors.
Eur J Cancer 3 183-191 (1967).

(244) Levan A, Manolov G and Clifford P.
Chromosomes of a human neuroblastoma: a new case with accessory minute chromosomes.
J Natl Cancer Inst 41 1377-1387 (1968).

(245) Crossen PE, Fitzgerald PH, Menzies RC and Brehaut LA.
Chromosome abnormality, megaloblastosis, and arrested DNA synthesis in erythroleukemia.
J Med Genet 6 95-104 (1969).

(246) Sandberg AA,Sakurai M and Holdsworth RN.
Chromosomes and causation of human cancer and leukemia.
Cancer 29 1671-1679 (1972).

(247) Biedler JL, Helson L and Spengler BA.
Morphology and growth, tumorogenicity, and cytogenetics of human neuroblastoma cells in continuous culture.
Cancer Res 33 2643-2652 (1973).

(248) Kovacs G.
Homogeneously staining regions on marker chromosomes in malignancy.
Int J Cancer 23 299-301 (1979).

(249) Pathak S.
Cytogenetic analysis of human breast tumors.
Cancer Genet Cytogenet 1 281-289 (1980).

(250) Barker PE, Lau Y-F, and Hsu TC.
A heterochromatic homogeneously staining region (HSR) in the karyotype of a human breast carcinoma cell line.
Cancer Genet Cytogenet 1 311-319 (1980).

(251) Mark J.
Double minutes - a chromosome aberration in Rous sarcomas in mice.
Hereditas 57 1-22 (1967).

(252) Levan G, Mandahl N, Bregula U, Klein G and Levan A.
Doudle minute chromosomes are not centromeric regions of the host chromosomes.
Hereditas 83 83-90 (1976).

(253) Levan G, Mandahl N, Bergtsson BO and Levan A.
Experimental elimination and recovery of double minute chromosomes in malignant cell populations.
Hereditas 86 75-90 (1977).

(254) Levan A and Levan G.
Large double minutes with ring-shape and rod-shape.
Hereditas 92 259-265 (1980).

(255) Nielsen K.
Chromosomal evolution in the Ehrlich-Lettre complex of hyperdiploid mouse ascites tumors:
Results from seven laboratory strains.
Hereditas 84 77-107 (1976).

ABBREVIATIONS

A	adenosine
AICAR	5-amino-4-imidazole-carboxamide
ALL	acute lymphoblastic leukemia
AMoL	acute monocytic leukemia
AUL	acute undifferentiated leukemia
bp	base pairs
C	cytosine
CAD	the multifunctional protein that catalyses the first three steps of UMP biosynthesis
CF	citrovorum factor or 5-formyl-THF
cpm	counts per minute
d	daltons
DAMPA	2,4-diamino-N^{10}-methylpteroic acid
DHF	dihydrofolate
DHFR	dihydrofolate reductase
dm	double minute chromosome
dpm	disintegrations per minute
DTT	dithiothreitol
F	folate
FdUrd	5-fluorodeoxyuridine
G	guanosine
GAT^-	a mutant cell line auxotrophic for glycine, adenosine and thymidine
Glu	glutamate
H	hypoxanthine
HAT	a selective medium containing hypoxanthine, aminopterin, thymidine and glycine

HPRT	hypoxanthine-guanine phosphoribosyl transferase
HSR	homogeneously staining region in trypsin-giemsa banded chromosome
Kb	kilobases or kilobase pairs
Kd	kilodaltons
5mC	5-methyl cytosine
MQ	methasquin
MTX	methotrexate
N	A,C,G or T
NOR	nucleolus organizing region
PALA	N-(phosphonacatyl)-L-aspartate
pDHFR 11 pDHFR 21	recombinant plasmids containing sequences complementary to mouse DHFR mRNA
poly A	polyadenylic acid
poly C	polycydidylic acid
Pu	purine
Py	pyrimidine
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
T	thymidine
THF	tetrahydrofolate
TS	thymidylate synthetase